The Regulatory Role of Non-Hematopoietic Bone Marrow Cells on Hematopoiesis in Steady-State and Inflammation

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Abbreviations

AEC	Arteriolar endothelial cell
AGM	Aorta gonad mesonephros
Ang1	Angiopoietin 1
BFU-E	Erythroid Burst Forming Unit
Bglap-Cre	Bone gamma carboxyglutamate protein
BM	Bone marrow
BMP	Bone morphogenetic protein
CAR	CXCL12 abundant reticular cells
Col2.3-Cre	Collagen 2.3
CD	Cluster of differentiation
CFU-G	Colony forming unit – granulocyte
CFU-GM	Colony forming unit – granulocyte macrophage
CFU-GEMM	Colony forming unit – granulocyte erythrocyte macrophage megakaryocyte
CFU-M	Colony forming unit – macrophage
cKit	c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CLC	Cardiotrophin like cytokine
CLR	C-type lectin receptors
CNTF	ciliary neurotrophic factor
Csf2	Conlony stimulating factor 2
Csf3	Conlony stimulating factor 3
CT1	Cardiotrophin-1
Cxcl4	C-X-C motif chemokine 4
Cxcl12	C-X-C motif chemokine 12 (SDF1)
Cxcr4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DNA	Desoxyribonucleic acid
EC	Endothelial cells
ET	Endotoxin tolerance
FACS	Fluorescence-activated cell sorting
Flt3(L)	FMS-like tyrosine kinase (ligand)
G-CSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GMP	Granulocyte monocyte progenitor cells
gp130	Glycoprotein 130

Gr1	Ly6G lymphocyte antigen 6 complex
HSC	Hematopoietic stem cells
HPC	Hematopoietic progenitor cells
HSPC	Hematopoietic Stem and Progenitor Cells
IGF-1	Insuline-like growth factor 1
IGFBP-3	Insuline-like growth factor binding protein 3
Infα	Interferon α
Infβ	Interferon β
Infγ	Interferon γ
IL	Interleukin
Lepr	Leptin receptor
LIF	Leukemia inhibitory factor
Lineage	collective term indicating terminally differentiated cells, in this study lineage cells are all hematopoietic cells expressing $CD3^+$,$CD4^+$,$CD8^+$,$CD11b^+$,$Gr\text{-}1^+$, $NK1.1^+$,$Ter119^+$, $B220^+$ and $IL\text{-}7R\alpha^+$ (Unless indicated)
LSK	$Lineage^-cKit^+Sca1^+$
LPS	Lypopolysaccharide
LT-HSC	Long-term hematopoietic stem cell
MEP	Megakaryocyte erythrocyte progenitor cells
mIL6R	membrane-bound IL6 receptor
MPP	Multipotent progenitors
MSC	Mesenchymal stem/stroma cells
MyD88	myeloid differentiation primary response gene 88
NLR	NOD-like receptors
OCL	Osteoclast
OSM	Oncostatin M
PAMP	Pathogen-associated molecular patterns
PαS	PDGFRα+ Sca1+
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGFRα	Platelet-derived growth factor receptor alpha
PDGFRβ	Platelet-derived growth factor receptor beta
poly I:C	polyinosinic-polycytidylic acid
PRR	Pattern-recognition receptors
qRT-PCR	Quantitative real-time polymerase chain reaction
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
SAA	Serum amyloid A

Sca-1	Ly6a lymphocyte antigen 6 complex
Scf	Stem cell factor
SEC	Sinusoidal endothelial cell
sIL6R	soluble IL6 receptor
SLAM	Signaling lymphocyte activation molecule
SLAM HSC	LKS $CD48^{-}$ $CD150^{+}$
SNS	Sympathetic nervous system
TGFβ	Tissue growth factor beta
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha
TPO	Thrombopoietin
Wt	Wild type

Summary

Hematopoiesis is the process of continuous generation of mature blood cells which all originate from hematopoietic stem and progenitor cells (HSPC). This process is taking place in the bone marrow (BM) where HSPCs are surrounded by different types of hematopoietic and non-hematopoietic cells which together form a complex supportive microenvironment. The enormous, balanced turnover of erythrocytes, platelets and leukocytes has to be tightly controlled as dysregulation can lead to pathological conditions such as aplasia or leukemia. Besides known intrinsic regulatory pathways, a growing body of evidence suggests that important extrinsic regulatory mechanisms have evolved to maintain hematopoietic homeostasis.

We wanted to investigate how the BM microenvironment supports the hematopoietic system during a stress situation, like in adaptation to severe systemic inflammation. Therefore, the principal goal was to get a deeper insight into the complex interactions between the hematopoietic system and its supportive microenvironment during demand-adapted hematopoiesis. Hence we wanted to find potental regulators of inflammation-driven hematopoiesis which are produced by the BM microenvironment. For this study, we injected wild type (WT) mice with lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (poly(I:C)) to mimic gram-negative bacterial or viral infection, respectively. We prospectively isolated different non-hematopoietic cell types from the BM according to their characteristic immunophenotypes (endothelial cells (ECs), mesenchymal stromal cells (MSCs), Cxcl12-abundant reticular cells (CARs) and a not further defined population called Unkn). We analyzed differential gene expressions among these different cell types and found that *Il6* expression was significantly and specificlly upregulated in CAR cells during LPS treatment. Therefore, we chose this gene for further analysis of its regulatory role during inflammation-driven hematopoiesis (see next paragraph). In addition, we wanted to generate whole genome expression profiles of non-hematopoietic cells of the BM microenvironment during steady-state, bacterial or viral infection, as well as during early development and ageing. To approach this aim in the best possible way, we optimized the isolation and preparation of the different non-hematopoietic BM cell types as well as advance the characterization of their immunophenotypes. Using the endothelial

cell marker endoglin (CD105) we are now able to distinguish arteriolar endothelial cells (AECs) and sinusoidal endothelial cells (SECs) within the endothelial cell population and therefore can sort one population in additon. We performed a first RNA sequencing run on one cohort of PBS, LPS and poly(I:C) treated mice and performed quality controls. As the prospective isolation protocol is now enhanced, and the controls of the first batch of sequenced data confirmed known gene expression, we are now confident in proceeding with prospectively isolating the different non-hematopoietic BM cell types from diverse conditions. Therefore, we will sort AECs, SECs, MSCs, CAR cells and Unkn fom young (15-18 days) and old (more than 2 years) WT mice as well as from PBS, LPS or poly(I:C) treated adult WT animals. Subsequently all samples willl be sequenced and we will be able to determine gene expression profiles followed by functional analysis of differential gene expression changes among them in future experiments.

We confirmed the high and significant upregulation of IL-6, specifically in CAR cells, after LPS stimulation by measuring IL-6 protein levels in plasma and BM lysates of reciprocal BM chimeric *Il6* $^{-/-}$ mice (*Il6* $^{-/-}$ → WT, WT → *Il6* $^{-/-}$) as well as in conditional *Il6*$^{-/-}$ mice (*Lepr-Cre;Il6* $^{fl/fl}$), in which IL-6 is specifically knocked out in CAR cells. Studying the hematopoietic response in LPS-treated *Il6*$^{-/-}$ and *Lepr-Cre;Il6* $^{fl/fl}$ mice, we observed a significant decrease in absolute cell numbers of cells of the myeloid lineage from the earliest HSPCs to mature myeloid cells upon chronic-repetitive LPS stimulation (9 injections over 3 weeks). Moreover, the total number of colony-forming units granulocyte (CFU-G) was significantly reduced in the BM of LPS-treated *Il6*$^{-/-}$ and *Lepr-Cre;Il6* $^{fl/fl}$ mice compared to their respective controls. Our data reveal that CAR cells play an essential role for a sustained hematopoietic response during chronic repetitive LPS treatment in producing high levels of IL-6.

Zusammenfassung

Hämatopoese ist der Prozess der kontinuierlichen Herstellung von gereiften Blutzellen, welche alle von hämatopoetischen Stamm- und Vorläuferzellen (HSPC) abstammen. Dieser Prozess findet im Knochenmark (BM) statt, wo HSPCs von verschiedenen hämatopoetischen und nicht-hämatopoetischen Zellen umgeben sind, welche zusammen eine komplexe, unterstützende Mikroumgebung bilden. Der enorme, ausgeglichene Umsatz von Erythrozyten, Blutplättchen und Leukozyten muss streng kontrolliert werden, da Störungen in der Regulation zu pathologischen Zuständen führen können wie Aplasie oder Leukämie. Abgesehen von bekannten intrinsischen regulatorischen Signalwegen, finden sich stetig neue Ergebnisse, die auf wichtige extrinsische regulatorische Mechanismen hindeuten, welche sich entwickelt haben, um das hämatopoetische Gleichgewicht aufecht zu erhalten.

Wir wollten untersuchen wie die Knochenmarkmikroumgebung das hämatopoetische System während einer Stresssituation, wie der Anpassung an eine schwere systemische Entzündung, unterstützt. Deshalb war unser Hauptziel ein besseres Verständnis der komplexen Interaktionen zwischen dem hämatopoetischen System und seiner unterstützenden Mikroumgebung während einer der Nachfrage angepassten Hämatopoese. Darum wollten wir potenzielle Regulatoren einer entzündungsgetriebenen Hämatopoese entdecken, welche von nicht-hämatopoetischen Zellen des Knochenmarks produziert werden. Für diese Studie wurden Wildtyp (WT) Mäuse mit Lipopolisaccharide (LPS) oder Polyinosinic-Polycytidylic acid (poly(I:C)) injiziert, um entweder eine gram-negative bakterielle oder eine virale Infektion zu simulieren. Wir isolierten verschiedene nicht-hämatopoetische Zelltypen aus dem Knochenmark anhand ihres charakteristischen Immunophänotypes (Endothelzellen (EC), mesenchymale stromale Zellen (MSC), retikuläre Zellen angereichert an Cxcl12 (CAR) und eine nicht weiter definierte Zellpopulation die wir darum Unknown (Unkn) nannten). Wir analyisierten dann differenzielle Genexpressionen von diesen verschiedenen Zelltypen und fanden heraus, dass IL-6 signifikant and spezifisch in CAR Zellen während der LPS Behandlung hoch reguliert wird. Deshalb wählten wir dieses Gen/Protein aus, um seine regulatorische Rolle während einer entzündungsgetriebenen Hämatopoese zu

untersuchen (siehe nächsten Paragraphen). Zusätzllich wollen wir auch Genomexpressionsprofile von nicht-hämatopoetischen Zellen aus dem Knochenmark generieren, während sich die Hämatopoese entweder im Gleichgewicht befindet, währed einer bakteriellen oder viralen Infektion, während der frühen Entwicklung oder im hohen Alter. Um dieses Ziel in der best möglichen Art und Weise in Angriff zu nehmen, mussten wir die Isolation und Präparation der verschiedenen nicht-hämatopoetischen Zelltypen aus dem Knochenmark optimieren und gleichzeitig auch die Charakterisierung ihrer Immunophänotypen verbessern. Wir haben nun die Möglichkeit arterielle Endothelzellen (AEC) und sinusidale Endothelzellen (SEC) innerhalb der Endothelzellpopulation zu unterscheiden, da wir den Endothelzellmarker CD105 benutzen und können damit noch eine zusätzliche Zellpopulation unterscheiden. Wir haben eine erste RNA Sequenzierung mit einer Kohorte von PBS, LPS und poly(I:C) behandelten Mäusen und entsprechenden Qualitätskontrollen durchgeführt. Da unser Isolationsprotokoll verbessert wurde und alle Kontrollen der ersten RNA Sequenzierung unseren Erwartungen entsprachen, können wir nun fortfahren mit dem Isolieren der verschiedenen nich-hämatopoetischen Zelltypen aus dem Knochenmark während verschiedenen Situationen. Wir werden AECs, SECs, MSCs, CARs und Unkn Zellen von jungen (15-18 Tage) und alten (mehr als 2 Jahre) WT Mäusen als auch von PBS, LPS oder poly(I:C) gehandelten adulten WT Mäusen isolieren. Anschliessend werden alle Proben sequenziert werden und wir werden bald die Möglichkeit haben Genexpressionsprofile zu ermitteln, welche wir dann auf differenzielle Genespressionen zwischen den verschiedenen Situationsbedingungen analysieren werden.

Es war uns möglich die signifikante Hochregulation von IL-6 in CAR Zellen nach LPS Stimulation zu bestätigen. Dafür massen wir die IL-6 Proteinspiegel im Blutplasma und in Knochenmarklysaten von reziproken Knochenmark Chimären von *Il6*$^{-/-}$ Mäusen (*Il6*$^{-/-}$ → *WT, WT* → *Il6*$^{-/-}$) als auch von gewebespezifischen *Il6*$^{-/-}$ Mäusen (*Lepr-Cre;Il6*$^{fl/fl}$) welche kein IL-6 in CAR Zellen generieren. Wir studierten die hämatopoetische Antwort auf eine LPS Stimulation in *Il6*$^{-/-}$ *und Lepr-Cre;Il6*$^{fl/fl}$ Mäusen und konnten einen signifikanten Rückgang der absoluten Zellzahlen von HSPCs über Vorläuferzellen bis hin zu reifen myeloischen Blutzellen nach chronischer, repetitiver Stimulation mit LPS (9 Injektionen über 3 Wochen) beobachten. Zudem waren die Gesamtzahlen von

koloniebildenden Einheiten von Granulozyten (CFU-G) im Knochenmark von LPS behandelten *Il6*$^{-/-}$ *und Lepr-Cre;Il6*$^{fl/fl}$ Mäusen im Vergleich zu ihren jeweiligen Kontrollen signifikant reduziert. Unsere Daten zeigen, dass CAR Zellen eine essentielle Rolle spielen im Aufrechterhalten einer hämatopoetischen Antwort während einer chronisch, repetitiven LPS stimulation, in dem sie hohe Mengen an IL-6 generieren.

Introduction

The general architecture and ontogeny of the hematopoietic system

The blood-forming or hematopoietic system that gives rises to all types of mature blood cells is maintained by a rare population of hematopoietic stem cells (HSCs) that reside at the top of this hierarchically constructed organ system. HSCs need to be preserved over a lifetime and this fundamental requirement is accomplished through their ability to self-renew. As the vast majority of mature blood cells, with the exception of some T cells and locally self-renewing macropages, are terminally-differentiated and post-mitotic with short half-lives they need to be constantly regenerated from HSCs to maintain homeostasis of the blood system. Therefore, besides self-renewing cell divsions, HSCs have the potential to divide and generate multipotent hematopoietic progenitors (HPCs) with limited self-renewal capacity but high proliferative potential, which in turn, will give rise to inceasingly lineage-committed progenitors finally generating all the various kinds of mature blood cells of the myeloid and lymphoid lineage (Figure 1). This enormous cellular amplification produces approximately $1x10^9$ erythrocytes and $1x\ 10^8$ white blood cells per hour in steady state in humans [1] (Nombela-Arrieta & Manz, 2017, accepted). As discussed below in more detail, the hematopoietic system is very flexible allowing it to react to hematopoietic challenges such as blood loss and infection with massively enhanced blood cell production (demand-adapted hematopoiesis) [2-5]. Hematopoiesis has to be tightly controlled by intrinsic regulatory pathways determined and operating within HSPCs (i.e. transcriptional programs executed by lineage-sprecific transcription factors) as well as extrinsic mechanisms, i.e. regulatory signals such as growth factors and cytokines released from other cell types within and outside of the BM that govern HSPC differentiation. The classical example of such extrinsic regulation originating outside of the BM cavity is erythropoetin produced by kidney cells. Erythropoetin is the primary stimulus for red blood cell production and hence its plasma levels are tighly controlled and inversely related to oxygen levles. Kidney cells can sense low oxygen pressure and as consequence start to increase erythropoietin production, which resutls in the stimulation of red blood cell production in the BM [6]. Incapability to maintain proper hematopoietic regulation can

lead to pathological conditions such as bone marrow failure or development of hematopoietic malignancies (myelodysplastic syndroms / leukemia) [7-13].

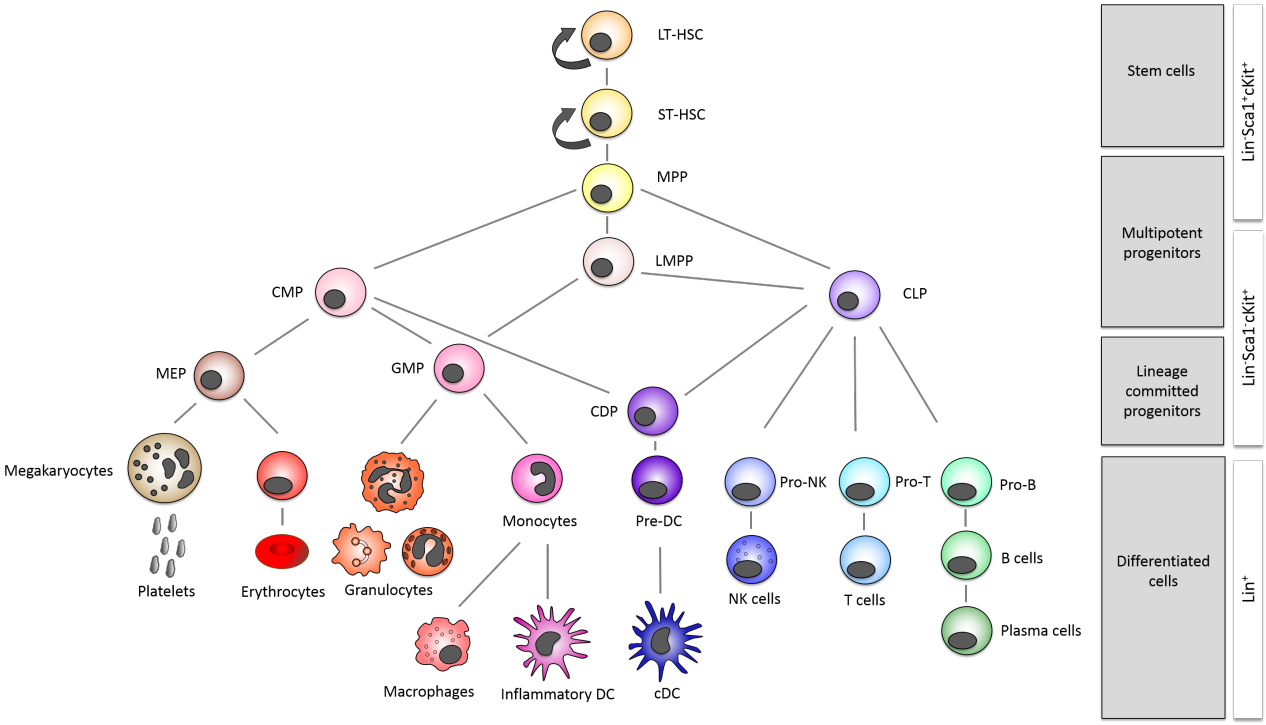

Figure 1: The hematopoietic hierarchy
The hematopoietic system is hierarchically organized and is maintained by HSCs which possess the abilty of self-renewal and multipotency. They then differentiate into multipotent progenitors with limited self-renewal capacity, which give rise to lineage committed progenitors which finally generate mature blood cells of all lineages.
Long-term HSC (LT-HSC), short-term HSC (ST-HSC), multipotent progenitor (MPP), common myeloid progenitor (CMP), lymphoid-primed multipotent progenitor (LMPP), common lymphoid progenitor (CLP), megakaryocyte–erythrocyte progenitor (MEP), granulocyte– macrophage progenitor (GMP), common dendritic progenitor (CDP), dendritic cell (DC), natural killer cells (NK cells)

Vertebrate ontogeny of the hematopoietic system has been studied best in mice. During mouse embryonic development hematopoiesis first originates in the yolk sac on embryonic day E7.5. This first wave of hematopoiesis is called “primitive” because it mainly produces red blood cells to support the increasing oxygen demand of the growing mouse embryo [14]. The next wave called “definitive hematopoiesis” produces fully functional HSCs with long-term, multilineage differentiateion capacities in the area surrounding the dorsal aorta termed the aorta-gonad mesonephros (AGM) region on E10.5 [3, 15, 16]. Thereafter, HSCs migrate from the AGM to the placenta and fetal liver by E11-E12 and from there hematopoiesis shifts to the spleen and thymus by E14 where it is still present several weeks postnatally [17]. By E18 HSCs start to repopulate the BM where they reside and maintain hematopoiesis throughout adult life [3, 14].

Technological advances, most importantly in flow-cytometry and immunefluorescence microscopy, have largely determined our increasing understanding of HSPC biology. Based on differential expression of cell surface markers it is possible to prospectively identify and isolate HSCs as well as multipotent progenitors and mature blood cells. HSC populations are routinely phenotypically defined by the absence of expression of markers of mature hematopoietic lineages (Lin^-) and the presence of Sca-1 c-Kit (LSK) in conjunction with the presence or absence of signaling lymphocytic activation molecule (SLAM) markers CD150 and CD48, respectively. The phenotype Lin^-Sca1^+ and $cKit^+CD150^+CD48^-$ is widely used to prospectively define and isolate LT-HSCs and is often referred to as LSK SLAM. Immunophenotypically-defined LT-HSCs (LSK SLAM) have a frequency of approximately 0.008% with regard to total BM cells correspondig to about 11'000 – 22'000 LT-HSCs per mouse [18-20]. It needs to be noted that, despite the possibilty to prospectively identify and isolate populations highly-enriched for HSCs by their phenotype, functional properties are required to define HSCs. The gold standard to prove HSC activity is the ability of long-term reconstitution and multi-lineage differentiation following transplantion into lethally irradiated mice. In this regard, a single transplanted HSC is capable of meeting this functional requirement [21-23].

A large body of evidence demonstrates that maintaining HSC quiescence is fundamental for HSC function, and indeed, snap shot analyses have shown that most adult HSCs are in the G0 phase of the cell cycle at any given time [24-26]. Hence, on average only 5-20% of HSCs are cycling at any given time and participate in blood cell production [24]. More detailed analyses, have shown that HSCs can be divided into two different functional groups of fast (5 or more divisions in 7 weeks) or slow dividing HSCs (no division within 12-14 weeks) [27, 28]. Since HSCs are mainly quiescent, multipotent and lineage-committed HPCs are largely responsible for the production of mature hematopoietic cells during unperturbed steady-state conditions [29, 30]. However, evolutionary pressure has shaped the hematopoietic system to possess remarkable adaptability to various stress situations such as bleeding and infection by enhanced proliferation and differention of both HSCs and HPCs with the overall goal to increase hematopoietic output according to increased demand [2, 4, 5].

Infection- and inflammation-driven Hematopoiesis

As mentioned above, severe systemic bacterial infection acts as a trigger to launch a hematopoietic response program – termed 'emergency hematopoiesis' – intended to augment numbers of innate immune cells, mostly granulocytes, to account for the increased demand for these cells during infection. Recognizing the dissemination of bacteria and switching steady-state to emergency hematopoiesis is a fundamental process. Any cell type that is able to sense life-threatening bacterial infection is required to be endowed with the molecular machinery to fulfill this function. This involves pathogen-sensing itself, i.e. the capability of detecting invading pathogens, but also translating this signal into enhanced hematopoiesis.

Pathogen-sensing is accomplished via pattern recognition receptors (PRRs) recognizing highly conserved pathogen-associated molecular patterns (PAMPs) which are often essential structural components of microorganisms such as lipoproteins and lipopolysaccharides (LPS). Several classes of PRRs exist including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) [31]. TLRs are the best-characterized family of PRRs [31, 32]. In light of the many cell types that have been reported to express TLRs, multiple, sometimes contradictory mechanisms of pathogen sensing followed by initiation of emergency hematopoiesis have been described. In this regard, both mouse [33, 34] and human [35-37] HSPCs express TLRs and it has been demonstrated that TLR stimulation promotes proliferation and myeloid differentiation *in vitro* and in *vivo* [34, 38]. While some data suggests that TLR stimulation on HSPCs induces proliferation and myeloid differentiation in a cytokine-independent manner [33], a recent study has revealed that HSPCs respond to *in vitro* TLR agonist treatment with secretion of inflammatory cytokines acting in an autocrine / paracrine manner [39]. However, the degree to which direct pathogen sensing by HSPCs is functionally relevant in comparison to other mechanisms remains to be determined (Figure 2) (discussed below).

According to the classic model of pathogen sensing and translation into emergency hematopoiesis, monocytes and macrophages, by virtue of their migratory behaviour or ubiquitious tissue

distribrution as well as expression of PRRs and subsequent production of inflammatory cytokines, are believed to serve as the primary pathogen-sensing cell types [40-45]. However, rigorous *in vivo* experimentation supporting this assumption is lacking. Indeed several lines of evidence demonstrated that mononuclear phagocytes are dispensable for initiating emergency granulopoiesis, at least during short-term LPS-induced inflammation [46]. In recent years, non-hematopoietic cells have gained broader attention as a putative candidate pathogen-sensing cell type based on data, demonstrating robust expression of TLRs on a variety of non-hematopoietic cell types such as endothelial cells (ECs) [47, 48] bladder epithelial cells [49], airway cells [50], and mesenchymal stromal cells (MSCs) [51-55]. It has been shown that the above-mentioned non-hematopoietic cells types participate in launching inflammatory responses following TLR stimulation [47-50, 55]. Along these lines, Boettcher et al. have recently undertaken a more systematic approach to dissect the relative contribution of hematopoietic vs. non-hematopoietic cells during pathogen-sensing and initiation of emergency hematopoiesis. Based on their initial study in which reciprocal *Tlr4*$^{-/-}$ BM chimeric mice were used to restrict TLR4 expression to either hematopoietic or non- hematopoietic cells, the authors unequivocally demonstrated that TLR4-expressing hematopoietic cells including monocytes and macrophages are dispensable for pathogen sensing during LPS-induced emergency granulopoiesis [46]. Unlike the popular assumption, *Tlr4*-expressing non-hematopoietic cells were critical for LPS recognition leading to granulocyte colony-stimulating factor (G-CSF) production and stimulation of emergency granulopoiesis. In a follow-up study, Boettcher et al. set out to identify the precise identity of this non-hematopoietic cell type by applying the Cre-loxP methodology to generate mice with a myeloid differentiation primary response gene 88 (*Myd88*)-deficiency in various candidate non-hematopoietic cell types, which by virtue of its function as adaptor molecule in TLR signaling abrogates LPS-mediated G-CSF secretion [56]. The authors were able to demonstrate that LPS-induced emergency granulopoiesis is primarily governed by G-CSF secreted from *Tlr4*-expressing ECs. Of note, *Csf3* (the gene name for G-CSF) induction following LPS stimulation was highest in BM ECs as compared to ECs isolated from other parts of the vasculature suggesting that BM ECs are functionally specialized essential regulators of emergency hematopoiesis. In conjunction with accumulating evidence from other studies [55, 57] these data point to a critical role of the

supportive BM microenvironment during the regulation of infection- and inflammation-induced hematopoiesis.

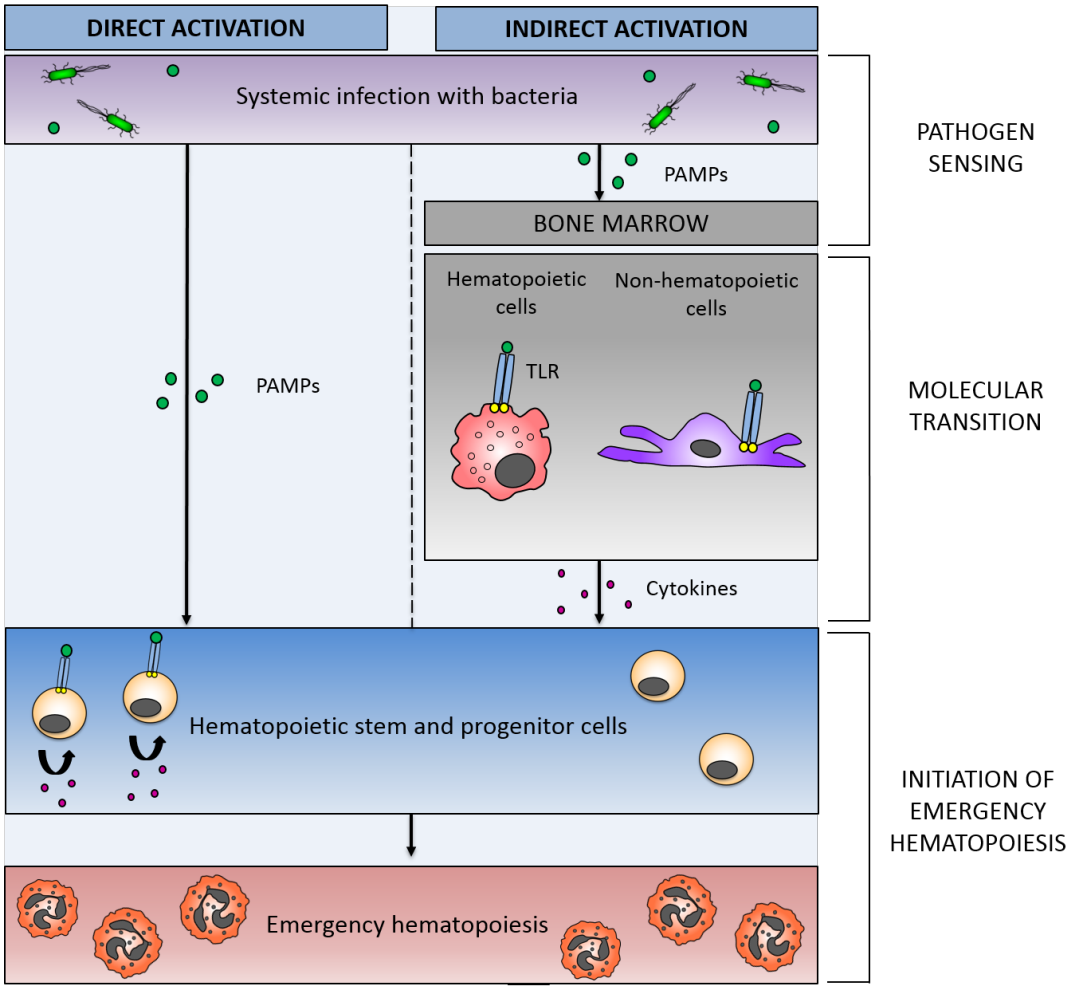

Figure 2: Direct and indirect activation of emergency hematopoiesis
Pathogens are sensed through pattern recognition receptors (PRRs) like Toll-like receptors (TLRs). These receptors are expressed directly on HSPCs, on other hematopoietic cells or on non-hematopoietic cells of the BM microenvironment. The binding of pathogen-associated molecular patterns (PAMPs) leads to a molecular transition. Subsequently, cytokines are released by the sensing cell or other cells and emergency hematopoiesis is activated.

The Bone Marrow Microenvironment – Concepts

The concept of a BM niche, i.e. a unique physical place within the microarchitecture of the BM cavity, where HSCs reside and develop into more mature hematipoietic cell types, was first proposed by Schofield four decades ago [58]. Schofield's proposal listed some basic concepts of a putative HSC niche: (1) a defined anatomic site, (2) a location where stem cells could be sustained and reproduce, (3) a place where differentiation was inhibited, (4) a limited space that also limited the numbers of stem cells.

Since then, the BM niche, also referred to as the BM microenvironment, has been studied extensively for its role in regulating hematopoiesis. The BM is located within the cavities of long and axial bones. The trabecular regions of the metaphysis where shown to mainly home transplanted HSCs rather than the epiphysis or diaphysis [59]. The outer region of the bone cavity forms the endosteal region which is mainly built by osteoblasts [60, 61]. Several studies have demonstrated, that HSCs with greater self-renewal capacity preferred to localize close to the bone surface rather than in central marrow regions [62, 63]. By contrast, other studies suggested a more random distribution of HSCs within the BM relative to the endosteum but consistenly demonstrated HSCs being close to vascular sinusoids [18, 64, 65]. These observations led to the proposal of differential niche models: the “endosteal niche” in which HSCs are residing in close proximity to the endosteum and in contact with osteoblasts [60, 61, 66] and the “vascular niche” in which cells are next to endothelial cells [18, 67]. Besides the distinction based on anatomic localization, it has been proposed that the endosteal and vascular niche differ functionally with respect to whether HSCs are quiescent or actively cycling, respectively [68]. More recent studies using state-of-the-art three-dimensional microscopy have challenged the existence of anatomically different niches but rather suggested a huge overlap [65, 69]. Notably, this is an active area of research and further studies are needed to provide definitive conclusions.

BM microenvironment – Cellular constituents

Multiple cell types of hematopoietic and non-hematopoietic origin participate in generating a supportive BM microenvironment (Figure 3). The evidence suggesting their role in regulating various aspects of hematopoiesis will be discussed below.

Non-hematopoietic cell types

Osteoblasts. The first evidence that osteoblasts regulate HSC function came from *in vitro* co-cultures of human $CD34^+$ HSPCs on human osteoblastic cells demonstrating that osteoblasts support the growth of HSPCs in short and long-term cultures [70]. Two seminal independent studies published in 2003 suggested that osteoblasts critically regulate stem cell function *in vivo*

via Jagged-1 and bone morphogenetic protein (BMP) signaling, respectively [60, 61]. However, more recent studies in which two extrinsically-operating factors known to maintain HSCs *in vivo*, C-X-C motif chemokine 12 (*Cxcl12*) and stem cell factor (Scf; also known as Kit ligand, *Kitl*) were conditionally depleted in mature osteoblasts using Bglap-Cre or Col2.3-Cre mice, respectively [71-73], showed normal BM cellularity and normal blood counts. Importantly, BM cells from these mice could still reconstitute lethally-irradiated mice. These data demonstrate that other cells than osteoblasts provide CXCL12 and Kitl necessary for HSC maintenance and suggest that osteoblasts may be dispensable for HSC maintenance, at least via provision of CXCL12 and Kitl.

Endothelial cells (ECs). With the discovery of the SLAM family markers to increase HSC purity it became possible to image HSCs within the BM and to analyze their localization and proximity to other cell types. Consequently, it has been shown that 60% of LSK SLAM (LT-HSC) cells were in contact with sinusoidal endothelial cells (SECs) [18]. These findings suggested a critical role of ECs in the regulation of HSC function which was later substantiated by data revealing that ECs are essential for HSC engraftment following chemotherapy- or irradiation-induced myeloablation [74]. Moreover, ECs produce several factors that are important for HSC self-renewal and differentiation such as Notch ligands and Kitl [73, 75]. In a more recent study, it has been revealed that actively cycling HSCs mainly associate with sinusoids while quiescent HSCs reside close to arterioles [68]. Collectively, it has become clear that cells of the vasculature are critically involved in the regulation of hematopoiesis.

Perivascular stromal cells. As mentioned above Cxcl12 is an important factor regulating BM homing and maintenance of HSCs. Using a Cxcl12-GFP reporter mouse in combination with imaging it could be demonstrated that most Cxcl12 is produced by a perivascular, reticular cell population termed Cxcl12-abundant reticular (CAR) cells to which 94% of HSCs are in close proximity [64]. CAR cells highly express Pdgfrα (CD140a) and Pdgfrβ (CD140b) but lack Sca-1 expression [76]. Importantly, when CAR cells were depleted from the BM, HSC numbers were reduced and HSCs were more quiescent highlighting the critical role of CAR cell-derived CXCL12 for HSC function [76]. Subsequent studies revealed that CAR cells, together with ECs, are also the

major source of Kitl [73, 76]. The Leptin receptor is also a marker for perivascular stromal cells and is not expressed by hematopoietic cells, bone-lining cells or endothelial cells [73]. $Lepr^+$ cells also express Pdgfrα and Pdgfrβ but not Sca1 and show a high expression of CXCL12 and Kitl and therefore highly match originally described CARs [77]. $Nestin^+$ BM cells define another perivascular cell type that is in close physical association with HSCs, produces high levels or HSC maintenance genes like CXCL12 and Angiopoietin1 (ANG1) and the deletion of $Nestin^+$ cells results in a significant reduction in BM HSCs. Moreover, $Nestin^+$ cells behave functionally as MSCs and can therefore differentiate into all mesenchymal lineages (osteoblastic, chondogenic and adipogenic) and show self-renewal potential expantion in serial transplantations [78].

Adipocytes. Older studies suggested that adipocytes are rather passive residents of the BM cavity [79]. A more recent study, however, compared hematopoiesis from adipocyte-rich vertebrae of the mouse tail and adipocyte-free thoracic vertebrae in their composition of hematopoietic cells and found that HSPC numbers as well as the CFU activity were reduced in adipocyte-rich BM. Using a mouse model in which adipogenesis is inhibited and therefore the mice are adipocyte-free revealed that the engraftment of HSCs from adipocyte-free mice was accelerated compared to WT controls and the colony forming activity was higher in mice lacking adipocytes [80]. Adipocytes were shown to secret factors like neuropillin-1 [81], lipocalin 2 [82, 83], adiponectin [84] and TNF-α [85, 86], which can impair haematopoietic proliferation. These data suggest that BM adipocytes act predominantly as negative regulators of hematopoiesis [80].

Nervous system. The BM is not only highly vascularized but also highly innervated by nerves from the sympathetic nervous system (SNS) and several lines of evidence demonstrate that the SNS is also involved in regulating hematopoiesis. In this light, it has been shown that the egress of HSPCs from the BM is dependent on signals from sympathetic nerves that regulate the CXCL12-CXCR4 interaction [87, 88]. Specifically, Mendez-Ferrer et al. were able to show that circadian fluctuations of noradrenaline secretion by SNS neurons activating the β3-adrenergic receptor on cells of the BM microenvironment regulates CXCL12 levels which, in turn, regulates BM egress of HSPCs [88]. Moreover, Yamazaki et al. [89] showed that transforming growth facor beta (TGF-β) is

a quiescence signal originating in the niche that controls HSC dormancy. TGF-β is under most conditions inactive (latent TGF-β) and needs to be activated to execute its function. In a subsequent study, the same authors elucidated that latent TGF-β is mainly processed into active TGF-β in a glial fibrillary acidic protein (GFAP)-expressing cell type. This GFAP-positive cells were identified as being nonmyelinating Schwann cells that ensheath sympathetic nerves in the BM and were in contact with a considerable proportion of HSCs [90].

Hematopoietic cell types

Megakaryocytes. It has been demonstrated that transplanted HSCs lodge close to megakaryocytes and that *in vitro* co-cultures of HSCs and megakaryocytes led to expansion of immunophenotypically-defined HSCs [91]. Notably, the expanded HSCs retained their reconstitution capacity and multi-lineage potential and the release of IGF-1 and IGFBP-3 from megacaryocytes was shown to be responsible for the increased expansion [91]. Later studies also showed that HSCs reside in close proximity to megakaryocytes and that the depletion of megakaryocytes affects the quiescent state of HSCs. Various soluble factors (Cxcl4, TGF-β1, Tpo) were proposed to be responsible for maintaining HSC quiescence [92-94].

Macrophages. Winkler et al. have been able to determine a role for BM-resident macrophages as important regulators of HSC maintenance via supporting the function of osteolineage niche cells, which produce HSC regulating factors such as CXCL12, ANG1 and Kitl. When G-CSF is used to mobilize HSCs from the BM to the PB, the number of BM macrophages is reduced leading to the suppression of osteolineage cells and subsequently to mobilization of HSCs to the periphery [95]. Similarly, Chow et al. showed that the depletion of BM macrophages decreases BM levels of CXCL12, leading to HSC egress [96].

Osteoclasts. Another class of hematopoietic cells that has become apparent as important niche component is bone-resorbing osteoclasts (OCLs) which control B-cell development in the BM by regulating the bone microenvironment and the fate of osteoblasts. An impaired activity of OCLs leads to decreased expression of CXCL12 and IL-7 in BM cells and a reduction in osteolineage cells [97]. Further results showed that ablation of OCLs leads to improper differentiation of

osteolineage cells into osteoblasts which impairs HSC homing [98].

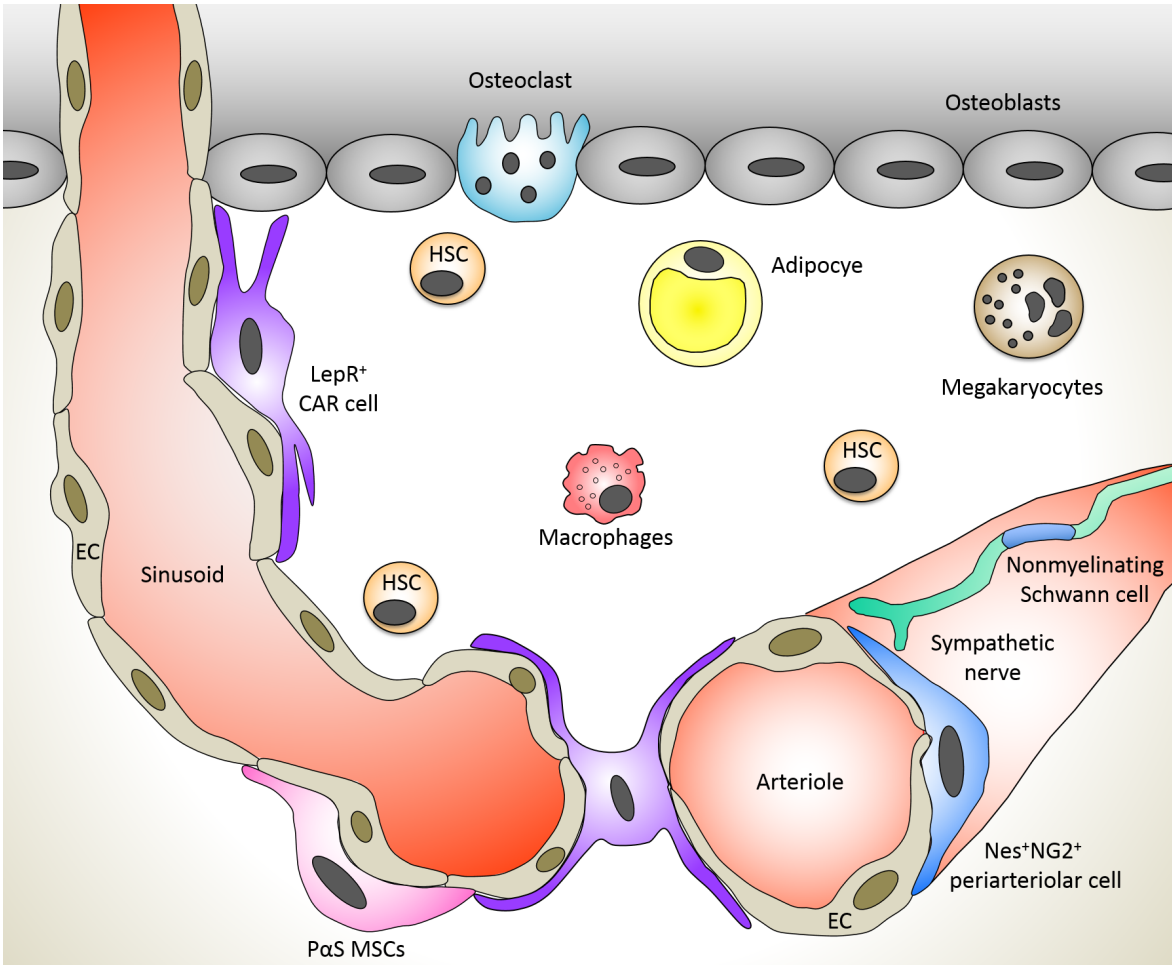

Figure 3: The bone marrow microenvironment
Different cell populations in the BM were reported to contribute to the regulation of hematopoiesis, some with a non-hematopoietic and others with a hematopoietic origin.
Hematopoietic stem cell (HSC), Cxcl12 abundant reticular cell (CAR), Leptin receptor positive cells ($LepR^+$), endothelial cell (EC), $Pdgr\alpha^+Sca1^+$ mesenchymal stromal cells(PαS MSC), Nestin and $NG2^+$ periarteriolar cells (Nes^+NG2^+)

Interleukin-6

Interleukin-6 (IL-6) is a multifunctional cytokine with various functions in hematopoiesis, the immune response and the acute phase response [99-101]. It is well known that IL-6 induces the maturation of B-cells into antibody producing plasma cells [102, 103], stimulates the differentiation of naïve $CD4^+$ T-cells into effector T-cell subsets [104-106] and is involved in the migration of neutrophils to the area of inflammation during the innate immune response [107-110].

Using IL-6 knock out mice, it was possible to study the role of IL-6 during steady-state

hematopoiesis as well as inflammation-driven hematopoiesis. While there was no obvious regulatory role of IL-6 during steady-state hematopoiesis, IL-6 knock out mice responded abnormally to myeloablation and anemia suggesting a role of IL-6 in HSPC proliferation and differentiation under regenerative conditions [111, 112]. In this regard, Zhao *et al.* have recently shown that HSPCs are able to directly sense Toll-like receptor (TLR) agonists *in vitro,* and subsequently produce various cytokines including IL-6. Under specific experimental conditions of irradiation-induced myeloablation, HSPC-derived IL-6 contributed to hematopoietic recovery in an autocrine / paracrine manner [39]. Furthermore, Schürch *et al.* demonstrated that during viral infection, $CD8^+$ cytotoxic T-cells (CTL) release INFγ leading to the production of IL-6 from BM MSCs which, in turn, stimulated enhanced monopoiesis [57].

Interleukin-6 signaling occurs through the heterotrimeric IL-6 receptor that consists of two functional subunits. The IL-6 receptor α chain acts as the ligand-binding subunit but lacks signal-transducing capacity. It was shown to be expressed on a few cell types, including macrophages, neutrophils and some T cell subsets [113-116]. The signal-transducing β subunit, also called gp130, is ubiquitously expressed and not only binds IL-6 but is also a receptor subunit for IL11, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin like cytokine (CLC) and IL-27 [117]. IL-6 first binds the membrane-bound IL-6 receptor α subunit (mIL6Rα). Hereafter, the IL-6:IL6-Rα complex binds to two molecules of gp130 triggering IL-6 signal transduction and the activation of downstream pathways such as the JAK/STAT, the RAS/MAPK or the PI3K pathway [113-116]. This mode of IL-6R signaling is referred to as *cis*-signaling. Cells that do not express the IL-6 receptor α subunit but only the β subunit are primarily unresponsive to IL-6 [118]. However, the IL-6Rα subunit not only exists as membrane-bound version but also in a soluble form (sIL6R). Therefore, IL-6 can bind the soluble IL-6 receptor α subunit and the IL6:sIL6Rα complex can then bind to gp130 on any cell, activating downstream signaling pathways. This alternative mode of IL-6R signaling is called *trans*-signaling [114, 119].

Aims of the study

As described above, recent studies have unequivocally demonstrated that the BM microenvironment plays key roles in regulating physiological steady-state hematopoiesis [18, 60, 61, 64, 72-78, 80, 87-90]. Furthermore, accumulating evidence suggests that the cellular crosstalk between HSPCs and the BM microenvironment in steady-state as well as during infection- and inflammation-driven hematopoiesis may be more complex than currently appreciated. Therefore, the principal goal of my PhD thesis was to get a deeper insight into the complex interactions between the hematopoietic system and its supportive microenvironment in steady-state and during demand-adapted hematopoiesis. To achieve this, I also wanted to better characterize the different celullar components of the BM microenvironment.

Aim 1: To *identify* potential regulators of infection- and inflammation-driven hematopoiesis produced by non-hematopoietic cells of the BM microenvironment.

We set out to investigate how the BM microenvironment supports the hematopoietic system in adaptation to inflammation. To this end, WT mice were stimulated with LPS or poly(I:C) to mimic gram-negative bacterial or viral infection, respectively. Different immunophenotypically defined non-hematopoietic BM cells were sorted using flow cytometry and differential gene expressions among these cells were examined using microarray analysis. This investigation revealed IL-6 as potential regulator of hematopoiesis during inflammation, and thus, IL-6 was chosen for further validation (see also Aim 3).

Aim 2: *To optimize* the characterization and isolation of non-hematopoietic cells of the BM microenvironment to assess global molecular signatures during development, aging and inflammation.

In addition to study the regulatory role of the BM microenvironment during inflammation-driven hematopoiesis we also wanted to study its role during aging and development of the hematopoietic system. This work is done in collaboration with Patrick Helbling from the group of Prof. Dr. Cesar Nombela-Arrieta. To obtain gene expression profiles of non-hematopoietic BM cells of these

different conditions, we planned to perform RNA sequencing for which high amounts of good quality RNA are required. Therefore, we first optimized the methods of isolation and preparation of the different non-hematopoietic cell types as well as advanced the characterization of immunophenotypes of BM microenvironment cell subsets. We are currently using the newly established protocol to isolate BM microenvironment cell types of LPS or poly(I:C) treated WT mice as well as of young and aged mice. Gene expression profiles of these cells will be determined using RNA sequencing. The newly established protocol for the isolation of cells of the BM microenvironment was also applied in aim 3.

Aim 3: To *validate* regulators of inflammation-driven hematopoiesis provided by non-hematopoietic cell types of the BM microenvironment. (Research article)

Having obtained evidence for IL-6 production by non-hematopoietic BM stromal cells in our initial microarray analysis we set out to confirm and potentially extend these findings. Thus, we treated WT animals with LPS or poly (I:C) to mimic gram-negative bacterial or viral infection, respectively. Different non-hematopoietic cell types of the BM microenvironment were flow cytometrically sorted according to our newly established protocols (aim 2). I then determined gene expression for a selection of candidate hematopoietic growth factors and inflammatory cytokines by using qPCR. In line with the previous microarray data, we could independently confirm inducible *Il6* expression in non-hematopoietic BM stromal cells. Consequently, we chose to further investigate the regulatory role of IL-6 on hematopoiesis during infection- and inflammation-driven hematopoiesis using a variety of experimental approaches.

Results I

Identification of potential regulators of hematopoiesis during inflammation

In order to get a deeper insight into the interactions between the hematopoietic system and its BM microenvironment during inflammation-adapted heamtopoiesis, we treated WT mice with PBS as control or with LPS or poly(I:C) to mimic gram-negative bacterial or viral infection, respectively. We then prospectively isolated different non-hematopoietic BM cell types according to their expression of the cell surface markers CD45, Ter119, Sca-1, CD31 and CD140b using flow cytometry. First, non-hematopoetic cells were defined by the absence of the expression of CD45 and Ter119 ($CD45^-Ter119^-$) and endothelial cellls could be defined by their expression of CD31 ($CD45^-Ter119^-CD31^+$). Based on the differential expression of CD140b along with Sca-1 it is possible to distinguish MSCs ($CD45^-Ter119^-CD31^-CD140b^+Sca\text{-}1^+$) [120], CARs ($CD45^-Ter119^-CD31^-CD140b^+Sca\text{-}1^-$) [55, 76] and a pupulation which is not further defined within the remaining $CD45^-Ter119^-CD31^-$ non-hematopoietic cells and therefore was called unknown ($CD45^-Ter119^-CD31^-CD140b^-Sca\text{-}1^-$) (Figure 4). RNA was extracted and cDNA synthesized from these cell populations followed by gene expression analysis using microarray analysis(Figure 5). Subsequently, we determined differential expression of genes between the three different conditions (PBS control, LPS and poly(I:C)) (fold change higher than 1.5, p-value lower than 0.5, signal intensity higher than 3). We mainly focused on uniquely, differentially expressed genes upon stimulation and some genes were identified as potential regulatory genes of inflammation-driven hematopoiesis due to their differential expression (Figure 6). Among these genes *Il6* showed one of the highest up-regulations among all examined genes. *Il6* upregulation was only detectable during LPS treatment and specifically in CAR cells which could be confirmed using qPCR (Figure 7). Therefore, IL-6 was chosen for further functional validation of its putative regulatory role during inflammation-driven hematopoiesis. All further data related to IL-6 are described in the “Resarch Article” section.

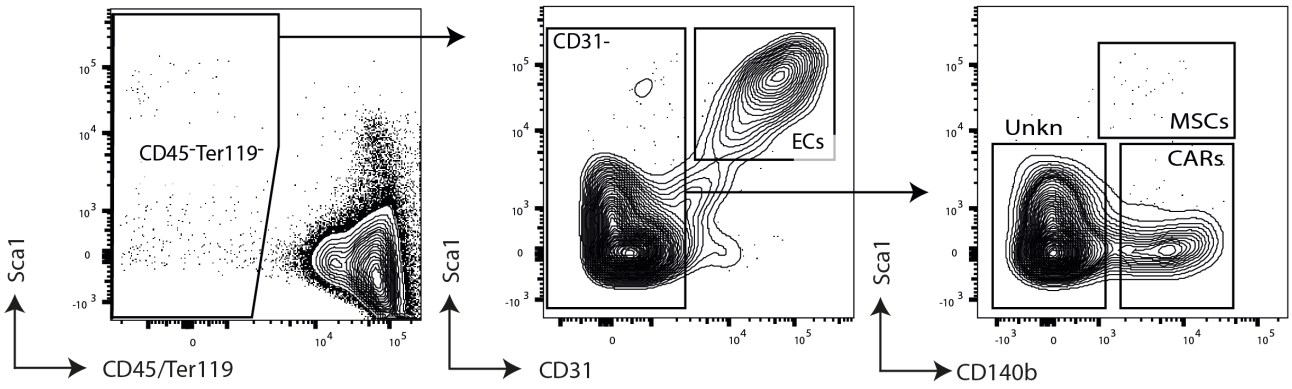

Figure 4: Representative FACS profile of the gating strategy of ECs, MSCs, CARs and

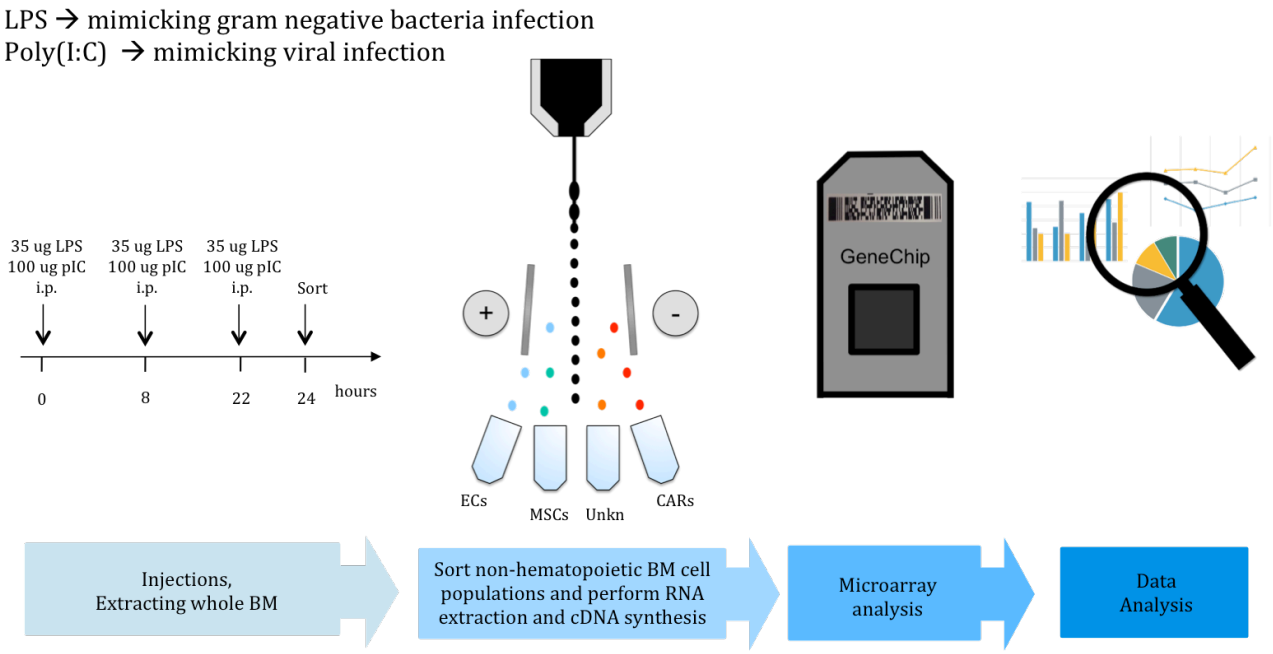

Figure 5: Project overview Microarray.
Non-hematopoietic BM cells of PBS, LPS or poly(I:C) injected WT mice were flow-cytometrically sorted, their RNA extracted and cDNA synthesized. The cDNA was then analysed using a microarray chip and differential gene expressions were examined.

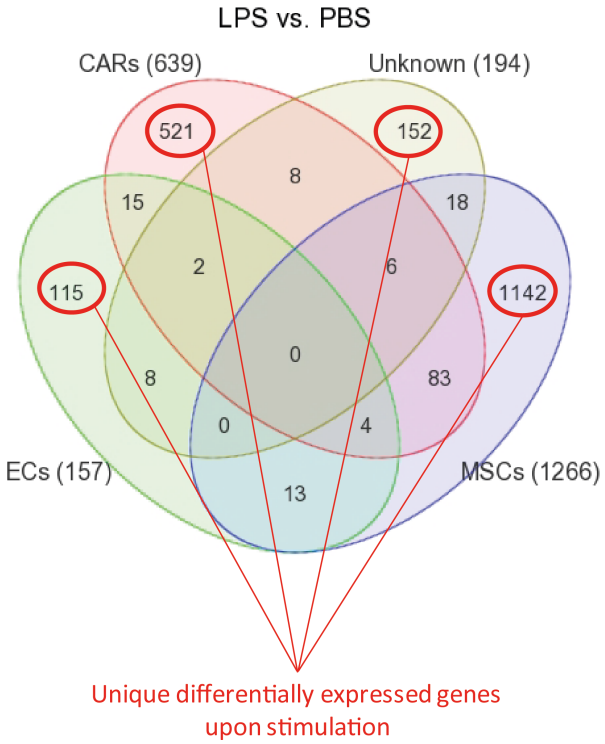

Figure 6: Data analysis of unique differentially expressed genes upon stimulation.
Gene expressions were compared between different treatment groups. Here an example is shown of genes that were differentially expressed between PBS and LPS-treated WT mice. We mainly focused on genes that were differentially expressed in one of the four analyzed cell populations.

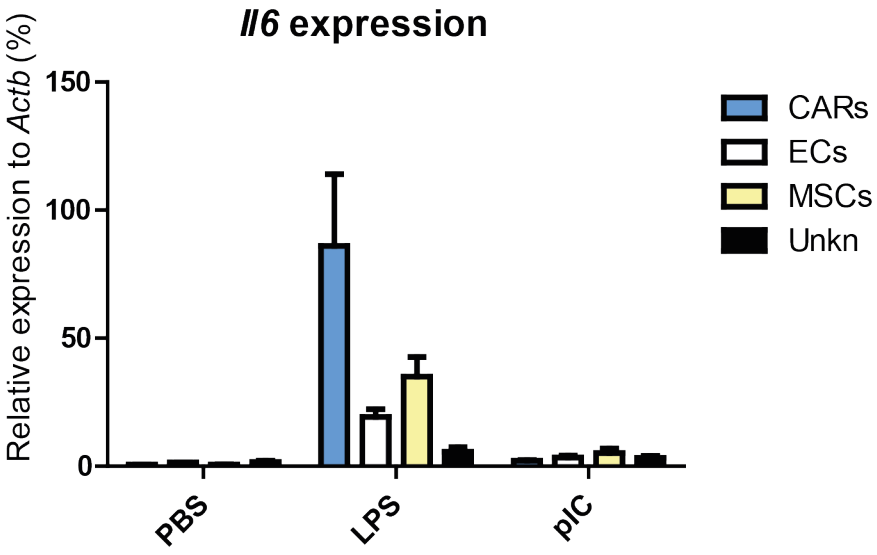

Figure 7: qPCR validation of the differential expression profile of *Il6*.
Examining gene expression profiles, we found a high upregulation of *Il6* and this specifically in CAR cells during LPS treatment. This could be confirmed using qPCR.

Changes in gene expression profiles of non-hematopoietic cells of the BM microenvironment during inflammation, development and aging

Based on the microarray analysis of non-hematopoietic BM cell subsets from LPS- or poly(I:C)-stimulated WT mice, we were able to study gene expression changes among these various populations in comparison to the steady-state situation, in order to identify potential regulators of inflammation-driven hematopoiesis. In addition, the group of Prof. Dr. Cesar Nombela-Arrieta followed the idea of analyzing gene expression changes of non-hematopoietic BM cells of young mice (15 – 18 days) compared to aged animals (more than 2 years). Therefore, we decided to collaborate and combine the two projects and investigate the gene expression changes of non-hematopoietic BM cells during the development of a mouse from short after birth over adulthood towards old age as well as during LPS and poly(I:C) treatment, simulating gram-negative bacterial and viral infection. We decided to analyze the gene expression changes using RNA sequencing, as this technique allows the unbiased detection of novel transcripts, whereas with microarray only known genes can be analyzed. Therefore, we decided to repeat the gene expression analysis of non-hematopoietic BM cells during LPS and poly(I:C) stimulation and used this opportunity to further optimize the characterization and isolation of non-hematopoietic BM cells to potentially get expression profiles of more refined cell populations (Figure 8).

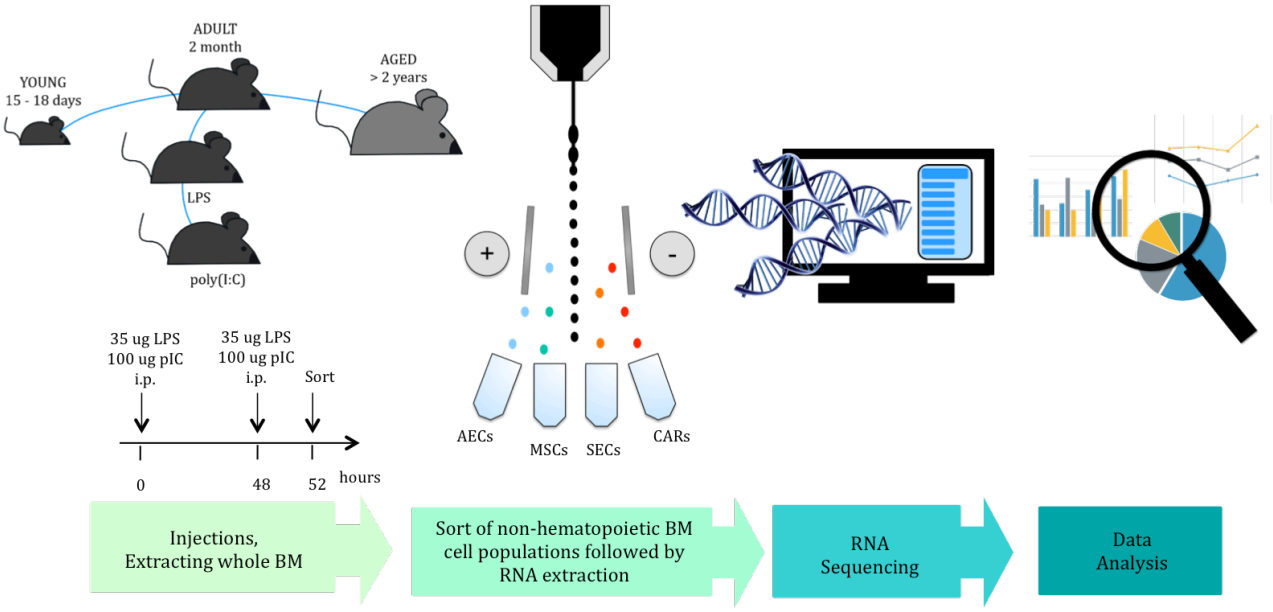

Figure 8: Project overview RNA sequencing.
BM of young, old and adult animals as well as of LPS or poly (I:C) treated adult mice will be processed. Different non-hematopoietic cell types will be sorted, their RNA extracted and gene expressions analyzed using RNA sequencing.

Characterization and isolation of non-hematopoietic BM cells

As we would need a higher amount of RNA for RNA sequencing as for the microarray analysis, we tried to improve our isolation protocol in order to extract higher cell numbers. We tested if mechanical treatment by crushing bones with a mortar and pestle would help to extract more cells from the bone, as this method was described before [120, 121]. However, we observed that crushing the bones in addition to just cutting them into small pieces led to suboptimal recovery of MSCs and CARs, hence we continued only cutting the bones (Figure 9). Given the non-hematopoietic cell populations only make up around 0.1-0.8% of the whole BM we decided to test enrichment methods. We compared two commercially available magnetic bead-based enrichment methods (MACS from Miltenyi Biotec and Easy Sep from Stemcell Technologies). Our findings revealed that the enrichment and consequently overall yield for the CD45/Ter119 negative fraction with the MACS columns was much higher than with Easy Sep method (Figure 10).

Using high-end imaging technologies it is possible to distinguish arteriolar and sinusoidal endothelial cells (AECs and SECs) within the EC population using the sinusoidal marker CD105, also called endoglin [122]. Therefore, we tested whether inclusion of CD105 into our FACS analysis would allow flow-cytometrically separation of AECs and SECs. Indeed, after gating on $CD31^+$ ECs we could distinguish two populations by gating on $CD105^{high}$ and $CD105^{low}$ expressing cells (Figure 11). Hence, our refined FACS strategy makes it possible to sort five non-hematopoietic BM cell populations including AECs, SEC, MSCs, CARs and Unkn (Figure. 12).

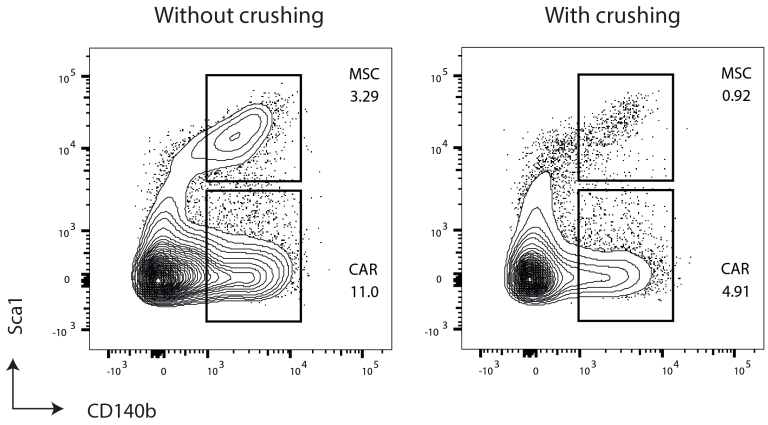

Figure 9: Representative FACS profile of Sca1^{+}CD140b^{+} MSCs and Sca1^{-}CD140b^{+} CARs after processing bones with or without crushing of bones (pregated CD45^{-}Ter119^{-}CD31^{-})

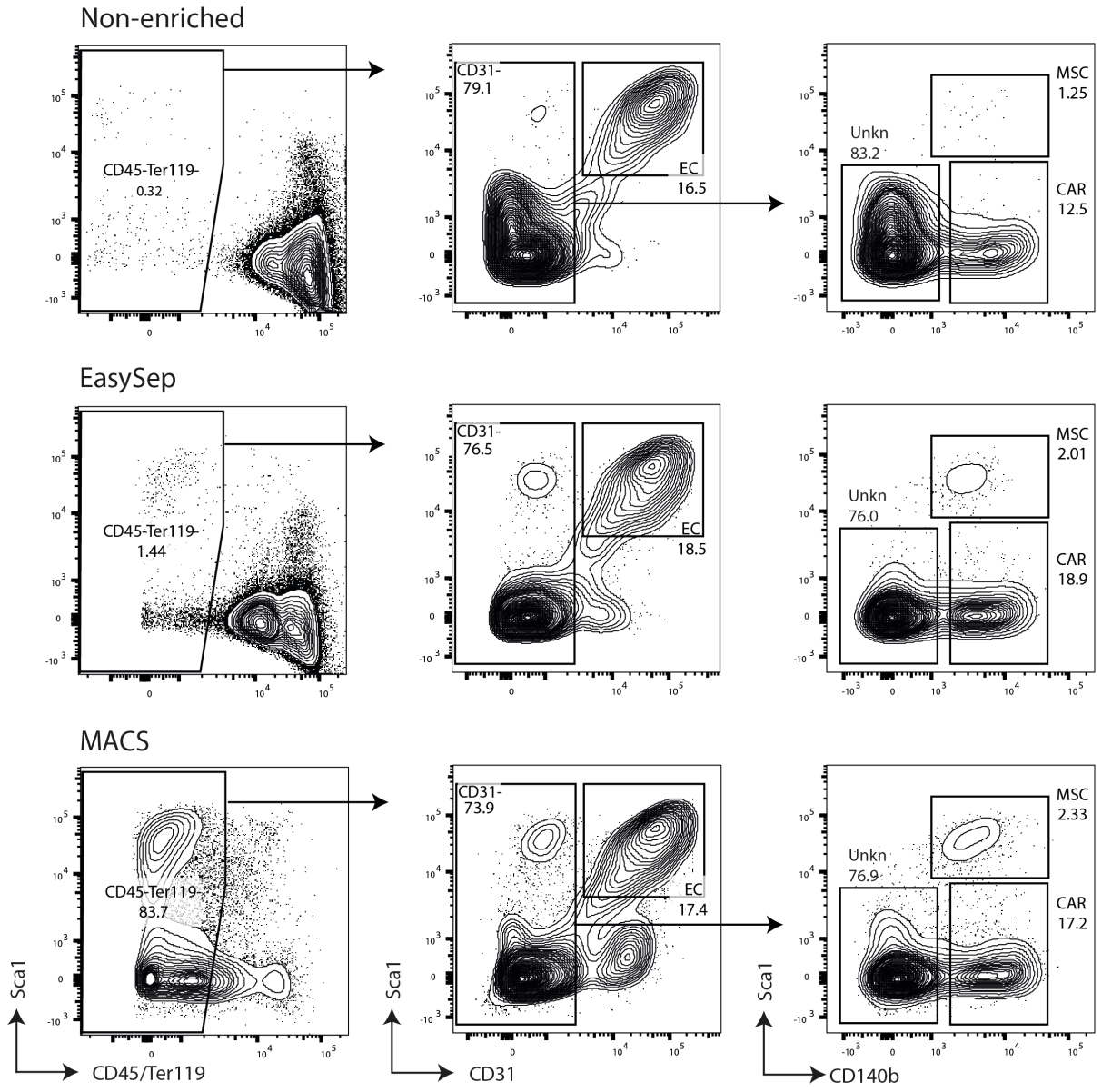

Figure 10: Representative FACS profile of the gating strategy of ECs, MSCs, CARs and Unkn without enrichment or using EaysSep or MACS columns.

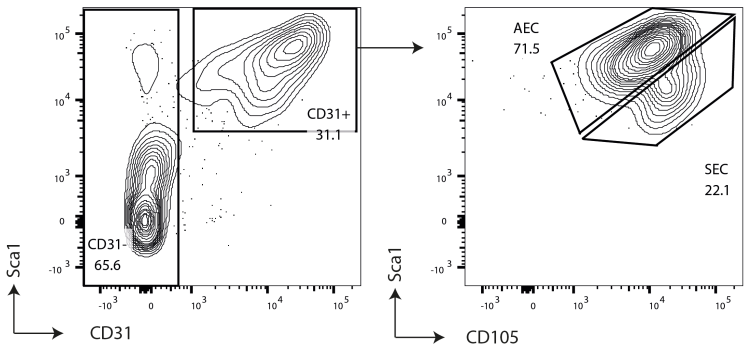

Figure 11: Representative FACS profile of the subdivision of AECs and SECs within $CD31^+$ endothelial cells (pregated $CD45^-Ter119^-$).

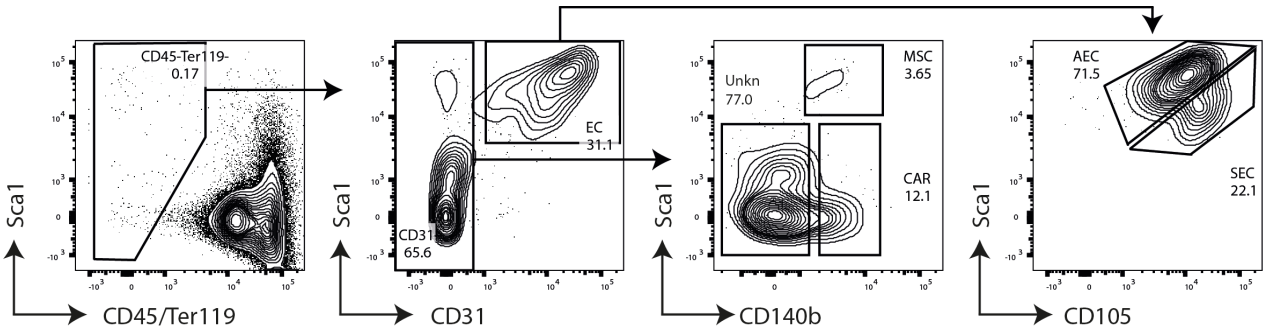

Figure 12: Representative FACS profile of the gating strategy of AECs, SECs, MSCs, CARs and Unkn.

Sorting of non-hematopoietic BM cells followed by RNA extraction

Given the relatively low frequency of non-hematopoietic BM stromal cells we first wanted to test whether it is feasible to purify enough RNA to subsequently perform standard library preparation without first having to amplify the RNA that inheres the potential disadvantage of leading to a biased representation of transcripts. In general, a total of 100 ng RNA per cell population is required to perform standard library preparation. Since a single cell usually contains 6-30 pg RNA, 3'300 – 16'600 non-hematopoietic BM cells per population would need to be isolated which could only be achieved by pooling mice for each condition. To test if this approach is feasible, we performed a pilot study to establish a protocol for RNA purification from limiting cell numbers. To this end, we first sorted splenocytes as there are abundant numbers contained in one mouse. We sorted different cell numbers and extracted the RNA from the samples using two different commercially available RNA extraction kits (RNeasy Micro and RNeasy Micro Plus). We observed better performance with the RNeasy Micro Plus Kit. However, total RNA amounts were considerably lower than predicted (Fig. 13) necessitating the use of amplification methods along with the library preparation. Accordingly, we decided to use the "Clonetech SMRTer Stranded totat RNA-Seq Kit for Pico Input Mammalian Total RNA" to prepare the RNA Seq library.

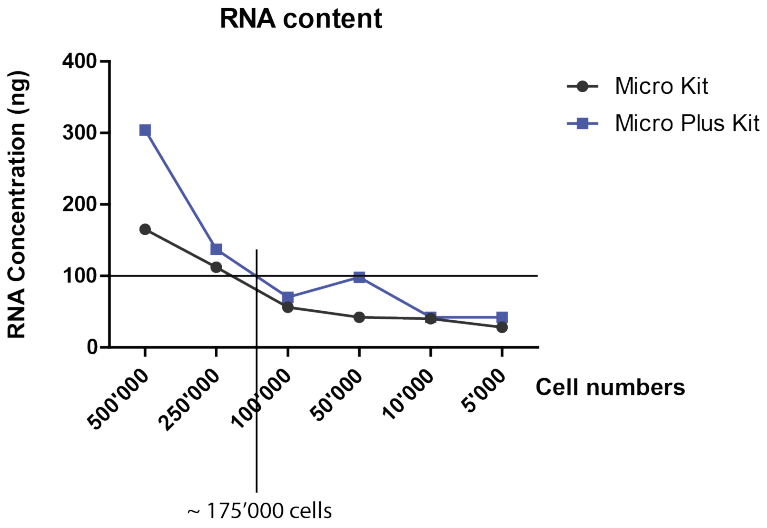

Figure 13: RNA amounts extracted from determined numbers of splenocytes with the RNeasy Micro or the RNeasy Micro Plus Kit and the cell numbers necessary to reach 100ng of RNA.

RNA Sequencing

In a first attempt to analyze the quality and quantity of sorted RNA for RNA sequencing, we injected one cohort of WT mice with PBS, LPS or poly(I:C). We sorted AECs, SECs, MSCs, CARs and Unkn from these different treatment groups and extracted their RNA using the commercially available RNeasy Micro Plus Kit. We measured RNA quantity and analyzed RNA quality by examining their RIN values. As quality and quantity of the total input RNA were suitable we used 600 pg of total input RNA of each sample to prepare an RNA seq library using the Clonetech kit for low input RNA. This kit includes cDNA synthesis of total input RNA followed by ribosomal cDNA depletion and PCR amplification of the low amount of input material. Subsequently, the library was sequenced and Patrick Helbling of the Nombela-Arrieta group and myself carried out quality controls. In order to test the accuracy of our flow-cytometry-based method for the isolation of non-hematopoietic BM stromal cell subsets, we analyzed expression levels of the sorting markers CD45, Sca1, CD105 and CD140b in the sorted populations, which showed the expected expression pattern (Figure 14A). In addition, we also analyzed the expression levels of different housekeeping genes to see if they are evenly expressed among all cell types in all conditions (Figure 14B). We also analyzed expression of *Kitl* and *Cxcl12*, as these genes show specifically restricted expression in ECs and CARs during steady-state hematopoiesis [64, 72, 73]. These expected expression patterns could be confirmed (Figure 14C). Moreover, we performed a principle component analysis (PCA) and created a dendrogram to investigate clustering of the different samples. These methods showed that samples of the same cell type of different conditions clustered as expected with the exception of AECs and SECs, which did not cluster independently (Fig. 15). This problem might arise from the fact that Sca-1 is an IFN-inducible gene and is up-regulated upon activation due to inflammation. Therefore, we will have to be more restricted in our gating of AECs and SECs for their flow-cytometrical sorting. However, another explanation could be that AECs and SECs are simply not so different form each other. In general, the RNA sequencing data gives us promising results and the confidence that the newly established protocol provides enough RNA with a good quality for RNA sequencing.

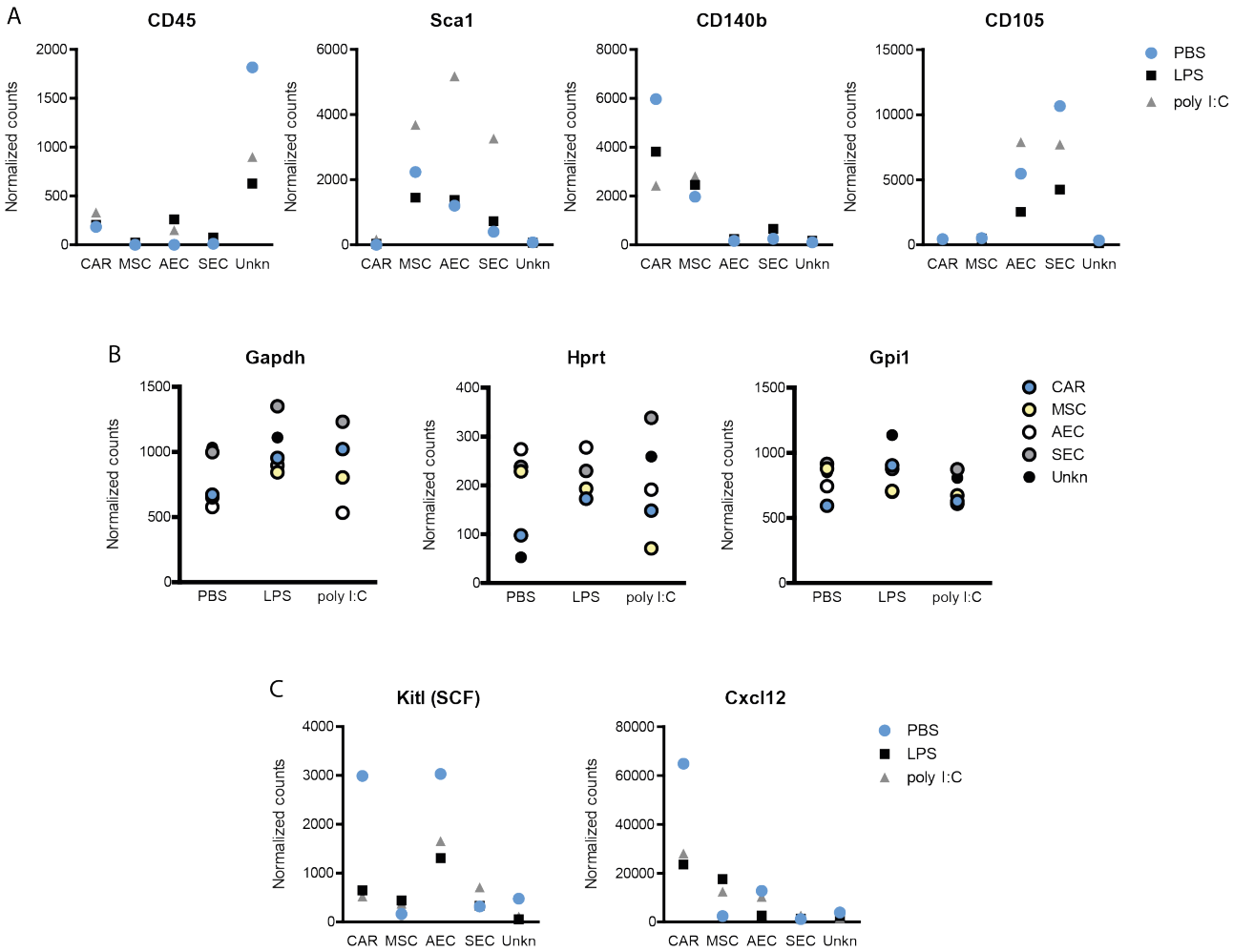

Figure 14: A) Normalized counts from RNA sequencing data of phenotypical markers of non-hematopoietic BM cell types, which were used for the prospective isolation of these cell types. In addition, the same normalized counts from RNA sequencing data are shown for B) some housekeeping genes and for C) Kitl and Cxcl12.

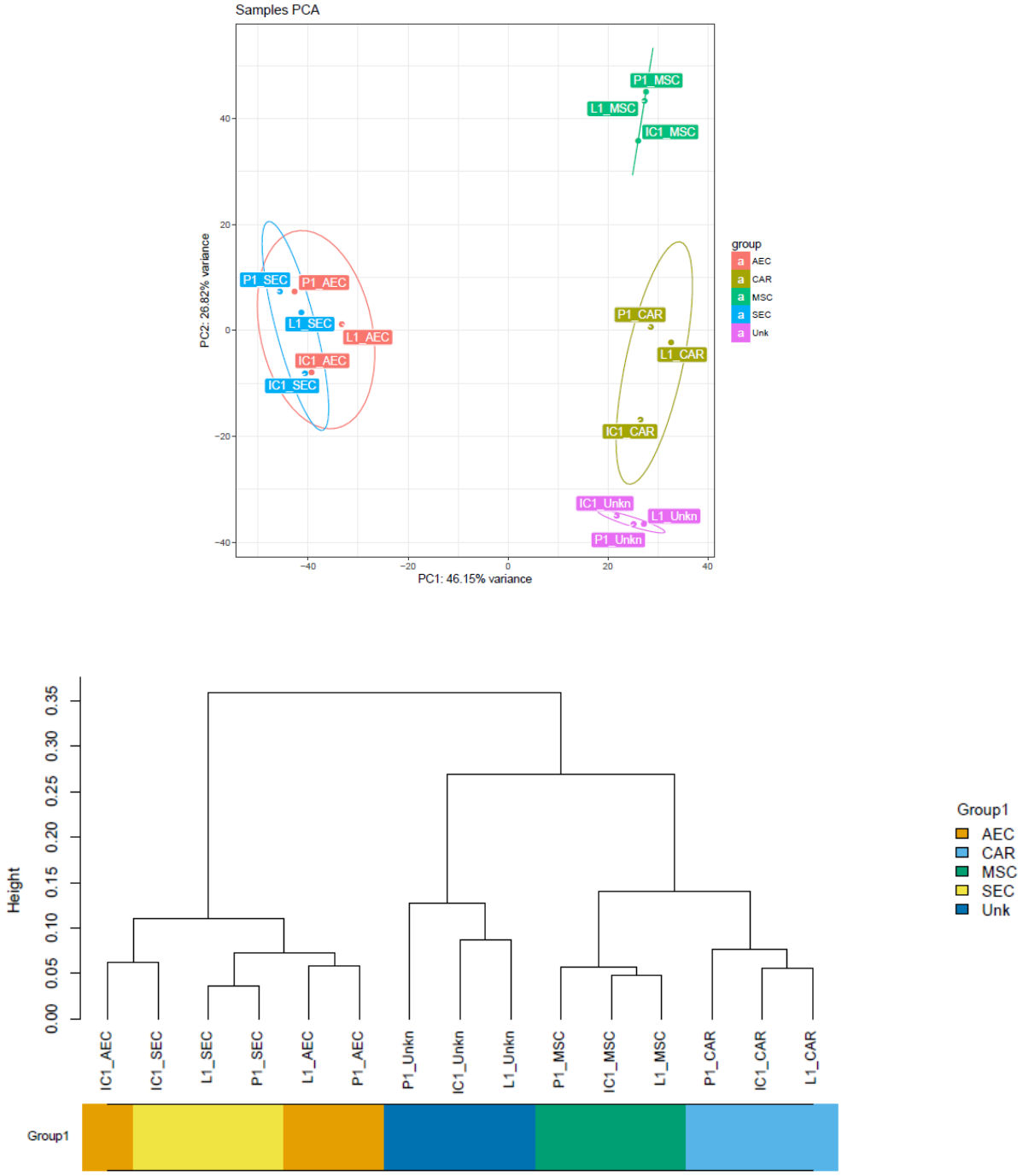

Figure 15: Principal component analysis and dendrogram of all analyzed genes, showing the dissimilarities of the non-hematopoietic BM cell subsets.

Results II

(Research Article in preparation)

Bone Marrow CXCL12-abundant Reticular Cells Are Key Regulators for a Sustained Hematopoietic Response During Chronic inflammation via Secretion of IL-6

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Abstract

Hematopoiesis is maintained by hematopoietic stem and progenitor cells (HSPCs) that are located in the bone marrow (BM) where they are embedded within a complex supportive microenvironment. The BM microenvironment not only regulates steady-state hematopoiesis but is also an emerging key player during emergency hematopoiesis. Through a combination of gene expression analyses in prospectively isolated non-hematopoietic BM cell populations and various mouse models we have revealed that BM CXCL12-abundant reticular (CAR) cells are a major source of systemic and local BM IL-6 levels following LPS stimulation. Importantly, while IL-6 is dispensable during the initial phase of LPS-induced emergency hematopoiesis, it is required to sustain emergency hematopoiesis during chronic-repetitive inflammation. Our data highlight the essential role of the non-hematopoietic BM microenvironment for the sensing and integration of pathogen-derived signals into sustained hematopoietic responses.

Introduction

The hematopoietic system is hierarchically organized and maintained by hematopoietic stem cells (HSCs) that give rise to highly-proliferative but increasingly lineage-committed hematopoietic progenitor cells (HPCs) which will eventually differentiate into all mature blood cell types [123]. This special functional architecture ensures massive cell amplification thereby accounting for the enormous cellular demand during steady-state hematopoiesis due to the short-lived nature of the vast majority of mature blood cells. It is estimated that approximately 0.5 - 1 x 10^{11} granulocytes are being generated per day during steady-state conditions in an adult human individual [1] (Nombela-Arrieta & Manz, 2017, accepted). Such remarkable proliferative and differentiation capacity requires intricate regulation as dysregulated hematopoiesis can lead to the inability to sustain life-long blood formation (bone marrow failure), and can cause disturbed differentiation (myelodysplasia) as well as excessive blood cell production (leukemia) [7-13].

The multifaceted nature of hematopoietic regulation is best studied for HSCs, in which a plethora of cell-autonomous regulatory mechanisms such as preferential use of specific DNA repair pathways and of particular metabolic pathways govern HSC self-renewal [124-126]. In addition, it has become increasingly evident over the past 15 years, that HSC-non-autonomous regulatory pathways operating within the bone marrow (BM) microenvironment and delivering regulatory signals to HSCs are essential to maintain HSC function throughout life [127]. The BM microenvironment consists of various mostly non-hematopoietic cell types including osteoblasts [60, 61], mesenchymal stromal cells [78], CXCL12-abundant reticular [64, 76] and perivascular stromal cells [72, 73], and endothelial cells [73-75, 128]. In addition adipocytes, sympathetic nerves, non-myelinating Schwann cells and also some hematopoietic cell types such as macrophages and megakaryocytes have been shown to be part of the supportive BM microenvironment [80, 87, 88, 90, 92-96].

Various naturally-occurring stimuli, most importantly bleeding and infection, have shaped the hematopoietic system during evolution to be able to rapidly augment its cellular output to meet the enormous demand for mature blood cells during these emergency situations [2, 4]. How infection- and inflammation-driven emergency hematopoiesis is regulated, is an active area of research and we have recently reviewed well-established and newer concepts around this theme

[5]. An emerging concept is that the BM microenvironment participates in regulating the switch from steady-state to emergency hematopoiesis. For example, we have recently shown that Toll-like receptor (TLR)-expressing endothelial cells sense pathogen-derived lipopolysaccharide (LPS) and translate the presence of gram-negative bacteria into emergency granulopoiesis via secretion of granulocyte colony-stimulating factor (G-CSF) [56]. However, the regulation of inflammation-driven demand-adapted hematopoiesis and the involvement of the BM microenvironment remain incompletely understood. Therefore, we have undertaken a systematic approach to gain a deeper understanding of how the non-hematopoietic BM microenvironment regulates the hematopoietic system's adaptation to severe infection with the goal to increase cellular output to meet the higher demand for mature myeloid cells. To this end, we performed gene expression analyses on prospectively isolated non-hematopoietic BM cells from LPS- and poly(I:C)-treated mice to mimic gram-negative and viral infection, respectively and found that Interleukin-6 (IL-6) is significantly and specifically up-regulated in CXCL12-abundant reticular (CAR) cells. Moreover, systemic and local BM IL-6 level largely depend on IL-6 production by CAR cells. Functional analyses using a variety of genetic mouse models elucidated that, although IL-6 is dispensable for a proper response during short-term LPS stimulation, IL-6 is critical to sustain emergency hematopoiesis during chronic-repetitive LPS-induced inflammation. Our results reveal a previously underappreciated regulatory role of the BM microenvironment as a central hub that controls the switch from steady-state to emergency hematopoiesis by virtue of its ability to sense systemically disseminated pathogens followed by the allocation of essential hematopoietic growth factors.

Material and Methods

Mice and generation of reciprocal chimeras

Mice used in this study included C57BL/6J (CD45.2$^+$), B6.SJL (CD45.1$^+$), *Tlr4*$^{-/-}$ (CD45.1$^+$ and CD45.2$^+$), *Rag1*$^{-/-}$ (B6;129S7-Rag1<tm1Mom>/J), *Il6*$^{-/-}$ (B6.129S2-Il6tm1Kopf/J) [111], *Lepr-cre* [129], *Il6*$^{flox/flox}$ [130] and *loxP-GFP* mice (B6.Cg-Gt(ROSA) 26Sor tm6(CAGZsGreen1)Hze/J). The *Il6*$^{flox/flox}$ mice were kindly provided by Dr. J. Hidalgo (Universitat Autònoma de Barcelona), *Tlr4*$^{-/-}$ were kindly provided by Dr. Shizuo Akira (Osaka University) and C57BL/6J (CD45.2$^+$) and B6.SJL (CD45.1$^+$) were obtained from Jackson Laboratories. Wt (CD45.1$^+$ and CD45.2$^+$), *Il6*$^{-/-}$ and *Tlr4*$^{-/-}$ (CD45.1$^+$ and CD45.2$^+$) mice were used to generate reciprocal chimeras. Mice were lethally irradiated with 950 cGy and transplanted intravenously with 5 × 10^6 total BM cells. All animals were maintained at the University Hospital Zurich animal facility and treated in accordance with guidelines of the Swiss Federal Veterinary Office. Experiments and procedures were approved by the Veterinäramt des Kantons Zurich, Switzerland.

LPS and pIC injections

In the short-term LPS induced inflammation studies, mice received 2 intraperitoneal injections of 35 µg ultrapure LPS from E. coli 0111:B4 (InvivoGen) 48 hours apart and were analyzed 24 hours after the last injection. For the long-term LPS induced inflammation studies, mice received 9 intraperitoneal injections of 35 µg ultrapure LPS from E. coli 0111:B4 (InvivoGen) over a periode of 3 weeks (day 0, 2, 4, 7, 9, 11, 14, 16, 18) and were analyzed 24 hours after the last injection (day 19). For some experiments, mice were injected once with 35 µg LPS or with 100 ug pIC (InvivoGen) and analyzed 4 hours later.

Flow cytometry

The following antibodies (all from eBiosciences, unless otherwice stated) were used to assess mature BM cell populations: anti-CD11b (M1/70), anti-Gr1 (RB6-8C5). anti-CD45.1 (A20, Biolegend) and anti CD45.2 (104) were used to analyze chimerism of reciprocal BM chimeric mice. To analyze HSPCs, the following antibodies were used; anti-CD3ε (145-2C11), anti-CD4 (GK1.5, Biolegend), andi-CD8α (53-6.7, BIolegend), anti-B220 (RA3-6B2), anti CD11b (M1/70), anti-Gr1

(RB6-8C5), anti-Ter119 (TER-119, Biolegend), anti-IL7Rα (A7R34), anti-NK1.1 (PK136, Biolegend), anti-Streptavidin Pacific Blue (Life Technologies), anti-cKit (2B8), anti-Sca-1 (D7, Biolegend), anti-CD34 (RAM34), anti-FcgR (93), anti-CD48 (HM48-1), anti-CD150 (TC15-12F12.2, Biolegend).

Classical and plasmacytoid dendritic cells were stained using anti-CD3e (145-2C11), anti-CD19 (MB19-1), anti-NK1.1 (PK136; Becton Dickinson), anti-CD45RA (14.8; BD Biosciences), anti-CD11c (N418), and anti-MHCII (M5/114.15.2).

Also used to sort different non-hematopoietic BM stromal cells were anti-Sca1 (D7, Biolegend), anti-Ter119 (TER-119, Biolegend), anti-CD45 (30-F11, Biolegend), anti-CD31 (390), anti-CD105 (MJ7/18), and anti-CD140b (APB5). To stain the Il6 receptor α and β subunits anti-CD126 (D7715A7, Biolegend)and anti-CD130 (KGP130) were used.

Processing of bones

Femurs, tibias and hip bones were removed, BM was flushed and cells were resuspended with digestion medium (DMEM/10% FCS/10mM HEPES/0.4% collagenase II (Worthington)/0.02% DNase I (Worthington)). Bones were cut into small pieces and combined with flushed BM in a total of 5 ml digestion medium. Bones and BM were incubated for 45 min at 37°C on a shaker and afterwards the cell suspension was filtered through a 70um cell strainer. LS MACS columns (Miltenyi Biotec) were then used to enrich the cell suspension for $CD45^{-}Ter119^{-}$ cells following the supplier's protocols.

Colony-forming unit (CFU) assay

$1x10^4$ red blood cell lysed whole BM cells of 3 week PBS or LPS treated mice were used. Cells were plated in a mix of methylcellulose (Methocult M3234; StemCell Technologies), Iscove's modified Dulbecco's medium (30% fetal calf serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol) and the following factors: mIL3 (10 ng/mL), hIL6 (10 ng/mL), mSCF (10 ng/mL), mGM-CSF (10 ng/mL), mTPO (10 ng/mL), mFLT3-Ligand (10 ng/mL) (all Pepro Tech), and hEPO (10 Ug/mL). Colonies were counted and classified after 12 days of in vitro culture.

For the serial replating of cKit enriched WT cells, LS MACS columns (Miltenyi Biotec) were used

for the enrichment. Cells were then plated in the same mixture of methylcellulose and cytokines as mentioned above. The only exception was that once hIL6 was added to the culture and once not. After 7 days of culture, colonies were counted and classified and the cells were resuspended using 37°C warm PBS and replated at the same conditions as before.

RNA extraction and cDNA synthesis

AEC, SEC, MSC and CAR populations were sorted directly into RLT Lysis Buffer provided by Qiagen (RNeasy Micro Plus Kit). Organs were removed and 30 µg per organ were snap frozen in liquid nitrogen. Using mortar, pistil and liquid nitrogen, organs were pulverized and transferred to RLT lysis Buffer of the RNeasy Mini Plus Kit (Qiagen). RNA was extracted following the suppliers protocols followed by cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative reverse-transcription PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR green reagent (Applied Biosystems). Samples were run on a 7500 FAST real-time PCR thermal cycler (Applied Biosystems) using *Gapdh* as housekeeping gene to normalize the RNA content of samples.

Enzyme-linked immunosorbent assay (ELISA)

Plasma samples were obtained by postmortem cardiac puncture. Blood samples were centrifuged and the supernatant was used for further procedures. BM samples were generated flushing one femur with a 1:1 mix of cOmplete ULTRA Tablets (Roche) dissolved in PBS and RayBio 2x Cell Lysis Buffer (Rybiotech, Inc.). IL-6 Protein levels were measured using an ELISA Kit (R&D) following the suppliers Protocol.

Equations and statistical analyses

Significance of differences was analyzed using Student t test. A difference between experimental groups was considered significant when the P value was 0.05. All statistical analyses were

calculated with Prism software (GraphPad Software, version 5).

Gene	Forward	Reverse
Cxcl12	GCATCAGTGACGGTAAACCA	GTTTAAAGCTTTCTCCAGGTACTC
Flt3l	AGTTCTGCTTCTCAGTCAGG	TTAGATATGCTGTACCCATCCTC
Csf3	GCTTCCTGCTTAAGTCCCTG	CTTAGGCACTGTGTCTGCTG
Csf2	CCTGTCACGTTGAATGAAGAG	TTGAGTTTGGTGAAATTGCCC
Il1a	CAACGGGAAGATTCTGAAGAAGAG	GGTTGGATGGTCTCTTCCAG
Il1b	TCTTTGAAGAAGAGCCCATCC	GTTGTTCATCTCGGAGCCTG
Il3	AGCTCCCAGAACCTGAACTC	ACTTCTCCTTGGCTTTCCAC
Il6	CTCTGCAAGAGACTTCCATCC	CGACTTGTGAAGTGGTATAGAC
Infa	GGACTTTGGATTCCCGCAGGAGAAC	GCTGCATCAGACAGCCTTGCAGGTC
Infb	AACCTCACCTACAGGGCGGACTTCA	TCCCACGTCAATCTTTCCTCTTGCTTT
Kitl	CAGAAACTAGATCCTTTACTCCTG	ACACTGACTCTGGAATCTTTCTC
Tlr1	CCTACAGAAACGTCCTATACCCA	CAGTAGAAGATGAGGGAATTTGGT
Tlr2	TCAGACAAAGCGTCAAATCTCAG	GCATCACATGACAGAGACTCC
Tlr3	GATAAAGCGAGTTTCACTTTCAGG	CAGATAGAGAACAGGTGCGT
Tlr4	CCAATGCATGGATCAGAAACTC	ATTTCACACCTGGATAAATCCAGC
Tlr5	ACACTTCAGCAGGATCATGG	ATAGTTGAAGCTGAGCAGGAG
Tlr6	AGGAAGTCTTGGTAAGTAAGAAGG	ATCCAAGCTCTATTTCCAGCAC
Tlr7	TTCAAGAAAGATGTCCTTGGCTC	AAATTTGTCTCTTCCGTGTCCA
Tlr8	CCTTCCTTTGTCTATAGAACATGG	ATGTGTAATGGCATTGTCTGAC
Tlr9	CCCAACATGGTTCTCCGTC	TCAGCTCACAGGGTAGGA
Tnf	CACGTCGTAGCAAACCACCAAGTGGA	TGGGAGTAGACAAGGTACAACCC
Tpo	GAACCCAGCTTCCTCTACAG	TGGGAACTTGTTTAGTGTGAGG

Results

Il6 expression is significantly and specifically induced in BM CAR cells upon a single LPS injection

In order to investigate mechanisms by which the non-hematopoietic BM microenvironment regulates the hematopoietic system's adaptation to severe systemic infection we modified a previously published method [120] to prospectively isolate different non-hematopoietic BM stromal cell populations by FACS. Based on expression of CD45 and Ter119 to exclude hematopoietic cells in conjunction with Sca-1, CD31, CD105 and CD140b, it was possible to flow-cytometrically define and isolate several non-hematopoietic cell types from mouse BM (Figure 1A). $CD45^-Ter119^-CD31^+$ endothelial cells, by virtue of their differential expression of Sca-1 and CD105 (endoglin), could be further delineated into sinusoidal endothelial cells (SECs, $CD45^-Ter119^-CD31^+CD105^{hi}Sca\text{-}1^{int}$) and arteriolar endothelial cells (AECs, $CD45^-Ter119^-CD31^+CD105^{int}Sca\text{-}1^{hi}$). The remaining $CD45^-Ter119^-CD31^-$ non-endothelial cells can be divided into the following three cell populations based on their CD140b (PDGFRβ) and Sca-1 expression: $CD45^-Ter119^-CD31^-Sca1^+CD140b^+$ and $CD45^-Ter119^-CD31^-Sca1^-CD140b^+$ cells which are highly enriched for mesenchymal stromal cells (MSCs) and CXCL12-abundant reticular (CAR) cells, respectively [76, 120]. The quintuple-negative ($CD45^-Ter119^-CD31^-Sca1^-CD140b^-$) population mostly consisted of debris as determined by morphological assessment of cytospin preparations and corroborated by the consistent failure to isolate RNA for expression analysis from this population (Figure 1A and data not shown). We therefore excluded this population from further analyses.

Having established this FACS-based prospective isolation methodology, wild-type (WT) mice received a single injection of high-dose lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (poly(I:C)) to mimic severe gram-negative or viral infection, respectively. Four hours after LPS or poly(I:C) injection, treated and PBS-injected control mice were sacrificed, the different non-hematopoietic cell populations were isolated by flow-cytometry and subjected to gene expression analysis by RT-qPCR for a selection of candidate hematopoietic growth factors and inflammatory cytokines (Figure 1B). Expression values relative to *Gapdh* were visualized as a heat map (Figure 1C) to better account for the huge range of expression values between weakly and strongly expressed genes. These experiments yielded several interesting pieces of information. First, most

of the assessed genes were neither relevantly expressed in PBS-injected, i.e. unperturbed steady-state hematopoiesis nor induced following LPS or poly(I:C) treatment. Second, in accordance with previously published data [73] steady-state *Kitl* (also known as stem cell factor, *Scf*) expression, although relatively weak, was specifically confined to AECs and CAR cells thereby confirming our prospective isolation strategy. Third, these results also confirmed and extend our previously published findings on *Csf3* (also known as granulocyte colony-stimulating factor, *Gcsf*) expression in endothelial cells upon LPS stimulation [56] by demonstrating that LPS-induced *Csf3* expression is largely restricted to AECs but not SECs. Most importantly however, among all assessed genes, *Il6* demonstrated the most remarkable expression pattern. While *Il6* was undetectable in steady state, it was highly significantly and quite specifically up-regulated in CAR cells following LPS but not poly(I:C) stimulation, and it also showed the highest expression compared to the other genes. To further investigate whether $CD45^{-}Ter119^{-}CD31^{-}Sca1^{-}CD140b^{+}$ cells are indeed highly enriched for CAR cells that are defined by their abundance for CXCL12 we independently assessed *Cxcl12* expression within the four different immunophenotypically-defined non-hematopoietic BM cell populations. As expected we observed extremely high *Cxcl12* expression in $CD45^{-}Ter119^{-}CD31^{-}Sca1^{-}CD140b^{+}$ cells (Figure S1A) which is compatible with the notion that this immunophenotype is able to define CAR cells [76].

In light of the above results, we focused our further efforts on studying the role of CAR cells as a potential relevant, previously unknown source of IL-6 during LPS-induced inflammation.

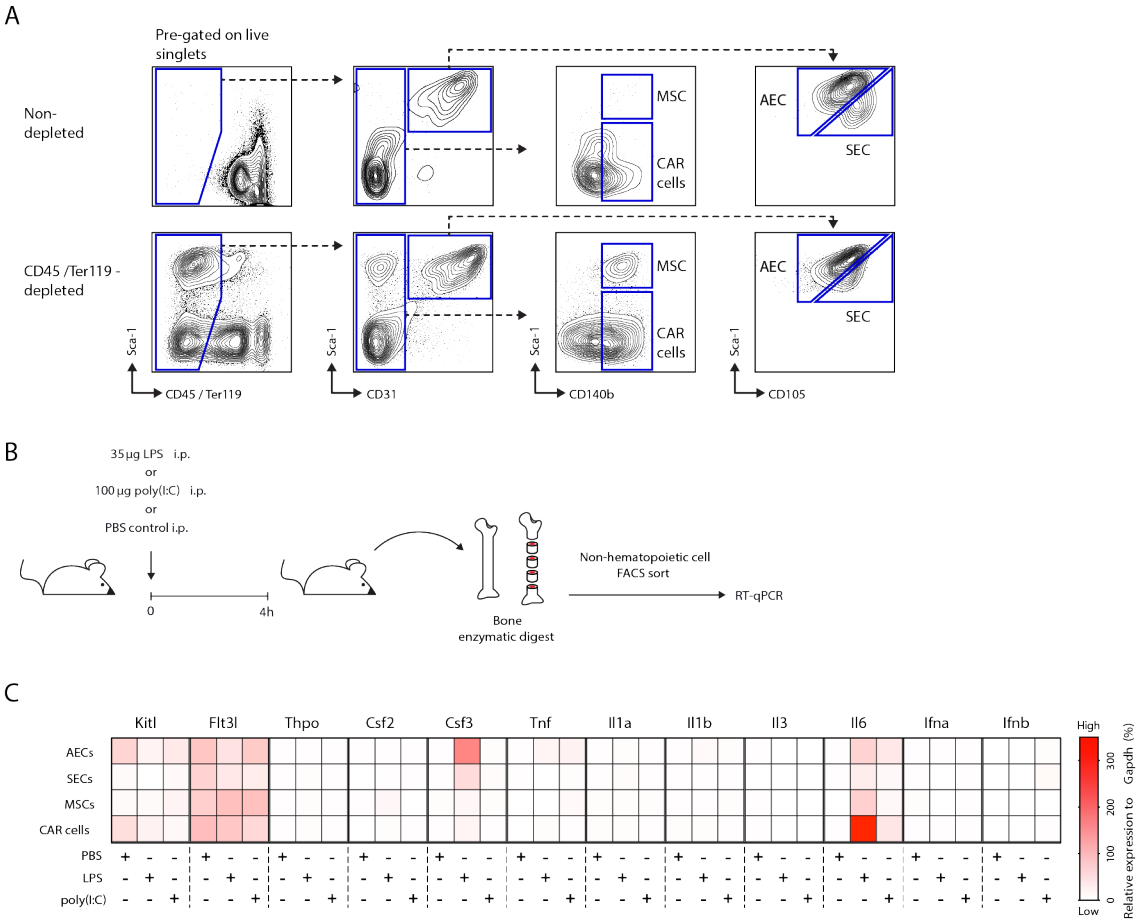
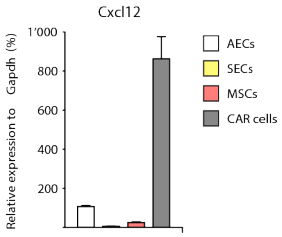


Figure 1. Expression of hematopoietic growth factors and inflammatory cytokines in non-hematopoietic BM stromal cell subpopulations following a single LPS injection.

(A) Representative FACS profiles showing non-hematopoietic cell types of the BM microenvironment either non-depleted (upper panel) or with MACS column-based CD45/Ter119 depletion (lower panel). AEC, arteriolar endothelial cells; SEC, sinusoidal endothelial cells; MSC, mesenchymal stromal cells; CAR cells, CXCL12-abundant reticular cells. **(B)** Graphical scheme depicting experimental outline for the induction of LPS- or poly(I:C)-induced inflammation and subsequent isolation of non-hematopoietic BM cells. **(C)** Gene expression of 12 hematopoietic growth factors and inflammatory cytokines from PBS-, LPS- or poly(I:C)-injected WT mice. Relative expression normalized to *Gapdh* from RT-qPCR is depicted as a heat map. *Kitl*, kit ligand, *Flt3l*, FMS-like tyrosine kinase 3 ligand; *Thpo*, thrombopoietin; *Csf2*, colony stimulating factor 2 (granulocyte-macrophage); *Csf3*, colony stimulating factor 3 (granulocyte); *Tnf*, tumor necrosis factor; *Il1a*, interleukin 1 alpha; *Il1b*, interleukin 1 beta; *Il3*, interleukin 3; *Il6*, interleukin 6; *Ifna*, interferon alpha; *Ifnb*, interferon beta.

A



Supplemental Figure 1. *Cxcl12* expression in different flow-cytometrically isolated non-hematopoietic BM cells.

(A) *Cxcl12* expression levels in BM AECs (white bar), SECs (yellow bar), MSCs (red bar) and CAR cells (grey bars) were assessed by RT-qPCR and normalized to house-keeping gene *Gapdh*. Results from three different experiments are shown.

CAR cells are the major source for systemic and local BM IL-6 levels upon LPS-induced inflammation

As an initial approach to reveal the relative contribution of CAR cells to IL-6 abundance following LPS-induced inflammation, we assessed *Il6* expression in total tissue extracts from major solid organs (heart, lung, liver, kidney, spleen) as well as total bone upon LPS stimulation. To this end, we injected WT mice with a single high-dose of LPS, sacrificed mice 4 hours later, isolated and homogenized tissues, and assessed *Il6* transcripts by RT-qPCR in comparison to PBS-injected control mice (Figure 2A). We chose this particular time point after a single high-dose of LPS for our initial analyses as plasma and BM IL-6 levels peaked at 4 hours post injection and rapidly returned to undetectable levels thereafter (Figure S2A and B). While *Il6* expression was undetectable in control mice, it was induced in all organs analyzed, albeit to a very different extent. Of note, inducible *Il6* expression was significantly highest in total bone as compared to all other organs. Spleen and lung had also relatively high *Il6* expression, whereas kidney, heart, and liver showed a minor *Il6* induction following LPS treatment (Figure 2B). These results strongly suggest that total bone consisting of both hematopoietic as well as non-hematopoietic cells is indeed a relevant source of IL-6 upon LPS-induced inflammation.

A common assumption infers that hematopoietic cells especially monocytes and tissue-resident macrophages are major sources of inflammatory cytokines. Moreover, a recently published study demonstrated that HSPCs are able to directly sense TLR agonist such as LPS and respond by secretion of inflammatory cytokines including IL-6, which, in turn, contributes to emergency hematopoiesis in an autocrine / paracrine manner albeit under very specific experimental conditions [39]. In order to dissect the relative importance of hematopoietic versus non-hematopoietic cells to IL-6 availability, we generated reciprocal BM chimeric mice with *Il6* expression restricted to either hematopoietic (WT ⇒ $Il6^{-/-}$) or non-hematopoietic cells ($Il6^{-/-}$ ⇒ WT) as well as control mice (WT ⇒ WT and $Il6^{-/-}$ ⇒ $Il6^{-/-}$) and assessed systemic and local IL-6 levels in their plasma and BM lysates following PBS or high-dose LPS treatment by ELISA (Figure 2A). We observed a strikingly clear pattern of highly and equally elevated plasma and BM IL-6 levels in LPS-treated WT ⇒ WT and $Il6^{-/-}$ ⇒ WT mice. By contrast, $Il6^{-/-}$ ⇒ $Il6^{-/-}$ and WT ⇒ $Il6^{-/-}$ mice were non- or severely hypo-responsive towards LPS stimulation in terms of elevation of IL-6 levels in

plasma and BM lysates (Figure 2C). Given the reported relative resistance to gamma-irradiation of tissue-resident macrophages, we have previously analyzed macrophage chimerisms in various organs of reciprocal BM chimeric mice 4 months after lethal irradiation and BM transplantation but found, with the exception of liver macrophages, negligible persistence of host-derived macrophage (<1%) [46]. Therefore, taking its limitations into account, BM chimeric mice are undoubtedly a valuable and well-suited experimental tool to generally address the question as to whether hematopoietic or non-hematopoietic cells contribute to a given phenotype. Furthermore, it has recently been shown that $CD8^+$ cytotoxic T cells stimulate IL-6 release from BM MSCs in an Interferon-dependent manner during viral infection [57]. To assess the role of T cells for IL-6 induction in the setting of LPS-induced inflammation, we also stimulated T cell-deficient *Rag1*$^{-/-}$ mice with high-doses of LPS and measured systemic and local BM IL-6 levels by ELISA which were indistinguishable between WT and *Rag1*$^{-/-}$ mice (Figure 2D). Thus, our data unambiguously demonstrates that T lymphocytes are dispensable for IL-6 induction following LPS-induced inflammation. Consequently, integrating the above results allows the conclusion that neither immature HSPCs nor mature hematopoietic cells (i.e. monocytes / macrophages or T lymphocytes) but instead non-hematopoietic BM cells are the most relevant source of IL-6, both systemically as well as locally in the BM, following LPS-induced inflammation.

Results from our gene expression analyses (Figure 1C) strongly suggest that CAR cells represent this BM-resident non-hematopoietic cell type responsible for IL-6 production upon LPS stimulation. In order to provide more evidence for this conclusion, we employed *Lepr-Cre* mice which have been shown to relatively specifically target perivascular non-hematopoietic BM cell populations largely overlapping with CAR cells [73, 77]. Breeding *Lepr-Cre* mice with *Il6*$^{fl/fl}$ mice [130] generated mice with an *Il6* deficiency in perivascular non-hematopoietic BM cells including CAR cells (*Lepr-Cre;Il6*$^{fl/fl}$ mice). Notably, LPS-induced elevation of IL-6 plasma and BM levels as measured by ELISA was almost completely abrogated in *Lepr-Cre;Il6*$^{fl/fl}$ mice, whereas control mice responded normally with an increase in IL-6 levels (Figure 2E).

In summary, by combining several independent experimental *in vitro* and *in vivo* models our data strongly suggests that, BM CAR cells are indeed the major source of IL-6 in plasma and BM following LPS-induced inflammation.

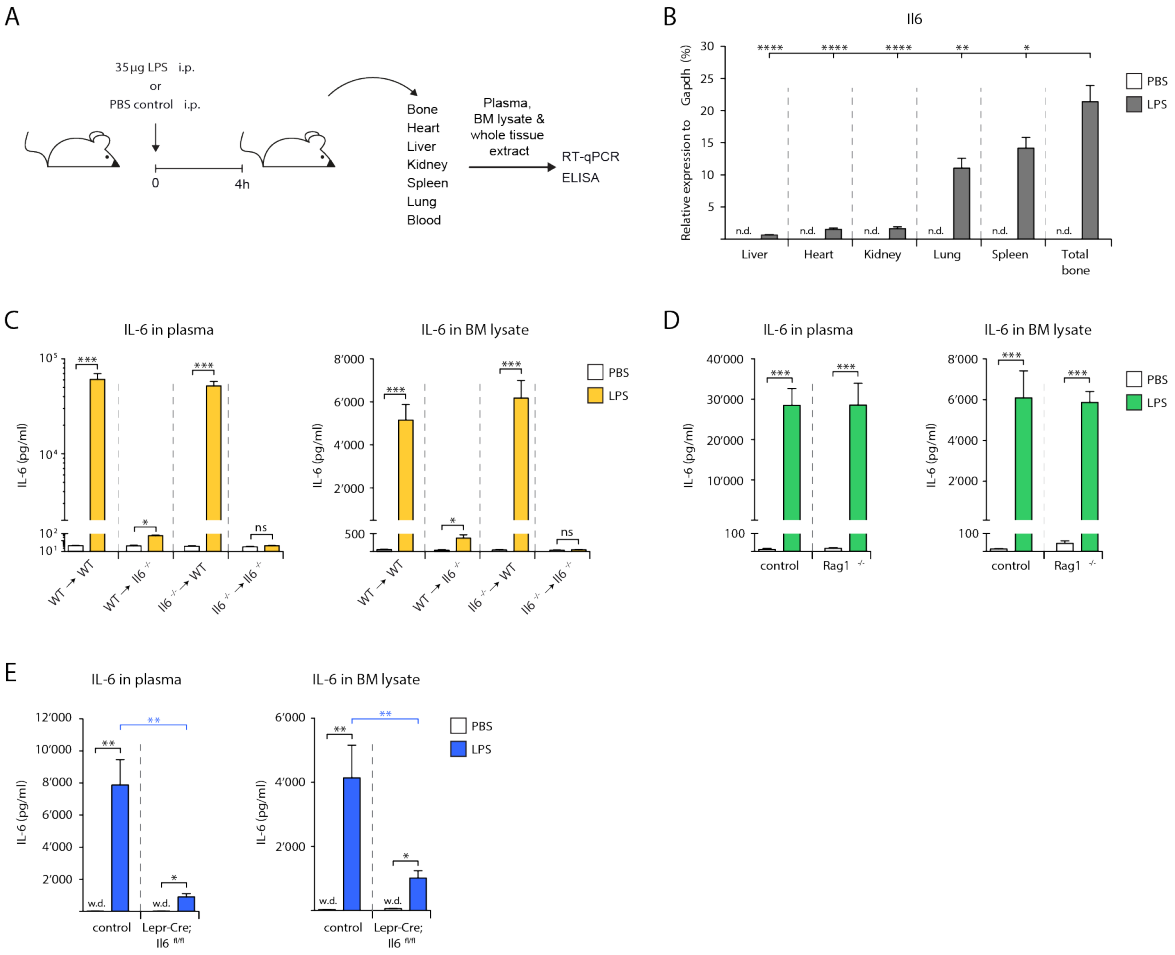
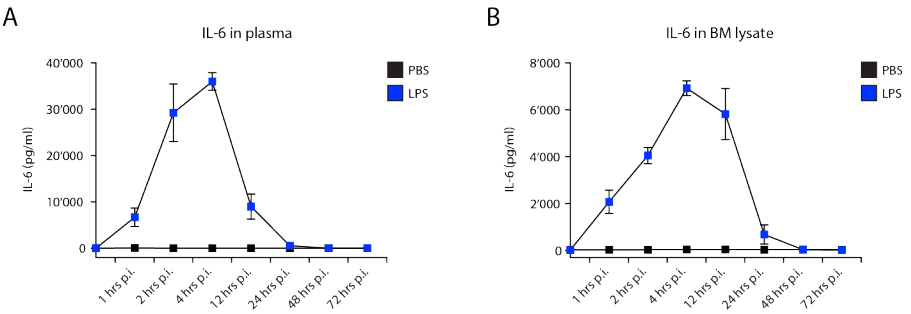


Figure 2. IL-6 expression in total tissue extracts from various organs and IL-6 levels in PB and BM lysates of reciprocal *Il6*$^{-/-}$ BM chimeric mice as well as *Lepr-Cre;Il6*$^{fl/fl}$ and *Rag1*$^{-/-}$ mice following a single LPS injection.

(A) Experimental outline illustrating induction of LPS-induced inflammation in WT, *Il6*$^{-/-}$ BM chimeric, *Rag1*$^{-/-}$, and *Lepr-Cre;Il6*$^{fl/fl}$ mice prior to isolation of major solid organs and collection of plasma and BM lysates for RT-qPCR and ELISA, respectively. This schedule is applicable to experimental data depicted in panels B – E. **(B)** *Il6* expression relative to *Gapdh* following LPS stimulation in whole tissue extracts from total bone, spleen, lung, kidney, heart, and liver of WT mice. **(C-E)** IL-6 protein levels in steady-state (PBS injected) plasma and BM lysates or after LPS stimulation in *Il6*$^{-/-}$ BM chimeric mice **(C)**, *Rag1*$^{-/-}$ mice **(D)**, and *Lepr-Cre;Il6*$^{fl/fl}$ mice **(E)**; nd, not detectable; wd, weakly detectable. (ns, non-significant; *, p=0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

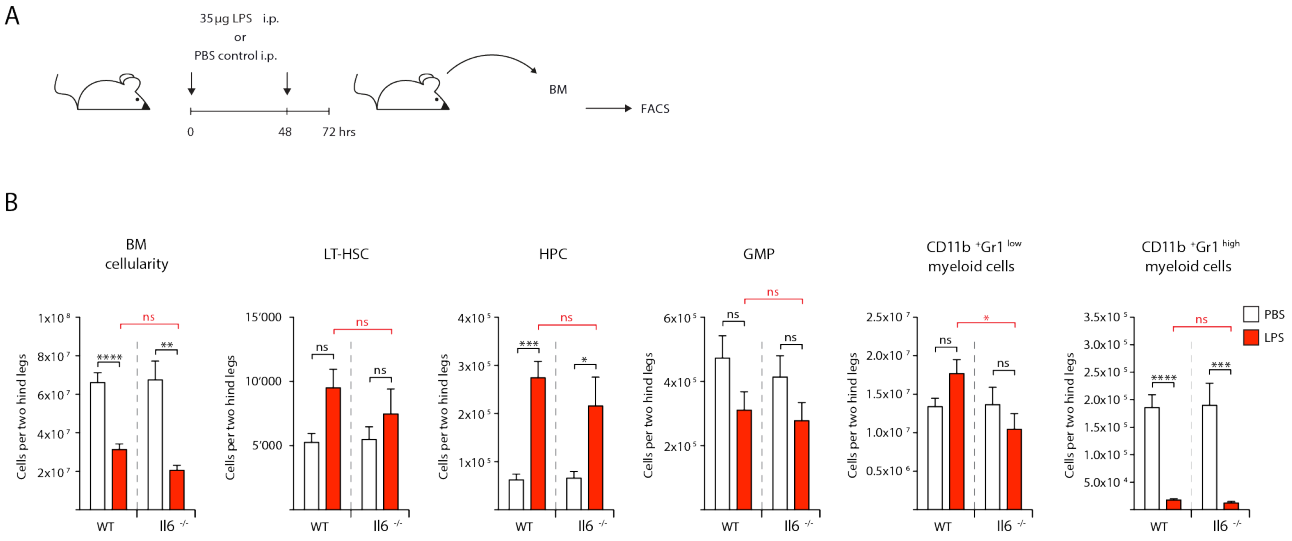


Supplemental Figure 2. Short-term kinetics of systemic and BM IL-6 levels following a single LPS injection.

(A) and **(B)** Time course over 72 hours of systemic and local BM IL-6 levels following a single LPS injection (blue square) into WT mice as compared to PBS-injected (black squares) control mice. Data from three independent experiments are shown.

IL-6 is dispensable for emergency hematopoiesis during short-term LPS-induced inflammation

Having revealed the primary source of IL-6 following LPS stimulation we set out to determine the functional impact of IL-6 on the course of LPS-induced emergency hematopoiesis. To this end, we used a well-established model of emergency hematopoiesis that we have previously shown to elicit a strong, primarily G-CSF-dependent hematopoietic response, i.e. *de novo* granulocyte production from upstream HSPCs [46, 56]. WT and *Il6*$^{-/-}$ mice were injected with two high-doses of LPS separated by a 48-hour interval, sacrificed 24 hours after the second LPS injection, and then analyzed for their HSPC and myeloid cell populations in BM by FACS (Figure S3A). As expected, there was a mostly significant change in the BM cellularity as well as absolute numbers of HSPCs and myeloid cell populations in BM of LPS-treated WT and *Il6*$^{-/-}$ mice as compared to PBS-treated WT and *Il6*$^{-/-}$ mice, respectively, indicating a physiological hematopoietic response towards LPS in both genotypes (Figure S3B and S3C). However, there were no statistically significant differences in the response pattern or magnitude between LPS-treated WT and *Il6*$^{-/-}$ mice. These results clearly demonstrate that IL-6 is dispensable for emergency hematopoiesis, at least during short-term LPS stimulation.



Supplemental Figure 3. Hematopoietic response in BM of *Il6*$^{-/-}$ mice following short-term LPS treatment.

(A) Graphical scheme depicting experimental outline for the induction of acute, short-term inflammation. **(B)** BM cellularity and absolute numbers per hind leg of immunophenotypically-defined LT-HSCs, HPCs, GMPs, CD11b^{+}Gr1^{high} and CD11b^{+}Gr1^{low} cells in LPS (red bars) or PBS (white bars) treated WT and *Il6*$^{-/-}$ mice according to the treatment schedule depicted under Fig. S3A. Data from three independent experiments are shown.

IL-6 is required to adequately increase numbers of immunophenotypically defined HSPCs and to maintain myeloid cell numbers during chronic-repetitive LPS-induced inflammation

In light of the above negative results for a role of IL-6 in the regulation of acute emergency hematopoiesis during short-term LPS stimulation, we hypothesized that IL-6 may have a regulatory function only during prolonged inflammatory conditions. Therefore, we subjected WT and *Il6*$^{-/-}$ mice to an LPS treatment scheme consisting of a total of 9 high-dose LPS injections over a period of 3 weeks followed by assessment of emergency hematopoiesis 24 hours after the last LPS injection (Figure 3A). BM HSPC and myeloid populations were defined by FACS (Figure 3B). Unlike short-term LPS stimulation (Figure S3B and S3C), chronic-repetitive LPS treatment led to significant differences between the responses observed in WT and *Il6*$^{-/-}$ mice. While chronic-repetitive LPS stimulation of WT mice leads to only a slightly but still significantly reduced BM cellularity, this decrease in BM cellularity is significantly more pronounced in *Il6*$^{-/-}$ mice (Figure 3C). Furthermore, although the percentages of BM HSPC and myeloid populations were similar in LPS-treated WT and *Il6*$^{-/-}$ mice (Figure S4), there was a significant, approximately 50% reduction in the LPS-induced increase in absolute cell numbers of Lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{-} long-term HSCs and Lin^{-}Sca-1^{+}c-kit^{+}CD150$^{+/-}$CD48^{+} hematopoietic progenitors (HPCs) in *Il6*$^{-/-}$ mice compared to WT mice (Figure 3D). In addition, Lin^{-}Sca-1^{-}c-kit^{+}CD34^{+}FcgR^{+} granulocyte-macrophage progenitors (GMPs), CD11b^{+}Gr1^{low} and CD11b^{+}Gr1^{high} myeloid cells which are largely equivalent to promyelocytes / myelocytes and mature neutrophils, respectively [131, 132], failed to increase or even decreased during chronic-repetitive LPS stimulation in BM of *Il6*$^{-/-}$ mice compared to WT mice (Figure 3D). In order to formally prove that CAR cell-derived IL-6 is responsible for the above-described failure in maintaining an appropriate quantitative hematopoietic response following chronic-repetitive LPS stimulation, we repeated the above experiment in *Lepr-Cre;Il6*$^{fl/fl}$ and control mice. As expected, we observed an identical response pattern after chronic-repetitive LPS treatment in *Lepr-Cre;Il6*$^{fl/fl}$ mice as compared to *Il6*$^{-/-}$ mice (Figure 3E and F).

Moreover, IL-6 levels in plasma and BM lysates of WT ⇒ WT and *Il6*$^{-/-}$ ⇒ WT mice subjected to chronic-repetitive LPS stimulation increased significantly and indistinguishable from each other (Figure 3G), whereas *Il6*$^{-/-}$ ⇒ *Il6*$^{-/-}$ and WT ⇒ *Il6*$^{-/-}$ mice were non- or severely hypo-responsive (Figure 3G). The same response pattern was also observed when *Lepr-Cre;Il6*$^{fl/fl}$ mice

underwent chronic-repetitive LPS stimulation (Figure 3H), indicating that even in the setting of prolonged LPS-induced inflammation systemic and local BM IL-6 levels depend on non-hematopoietic CAR cells. Notably, the overall magnitude of IL-6 production was substantially reduced after chronic LPS stimulation (Figure 3G and H) as compared to a single high-dose LPS injection (Figure 2C and E). Longitudinal measurements of plasma and BM IL-6 levels in WT mice during chronic-repetitive LPS stimulation revealed that, in fact, after an initial extremely high peak in plasma and BM IL-6 abundance, IL-6 levels remain significantly elevated throughout the 3-week period, however, at much lower levels (Figure S5). These results strongly suggest that maintenance of elevated IL-6 levels requires constant *de novo* IL-6 production in non-hematopoietic CAR cells whereas the initial IL-6 peak is presumably a consequence of a secretory burst from preformed IL-6 storage pools.

Collectively, our data demonstrates that CAR cell-derived IL-6 is required to adequately increase numbers of immunophenotypically defined HSPC populations and to maintain myeloid cell numbers during emergency hematopoiesis elicited by chronic-repetitive LPS-induced inflammation.

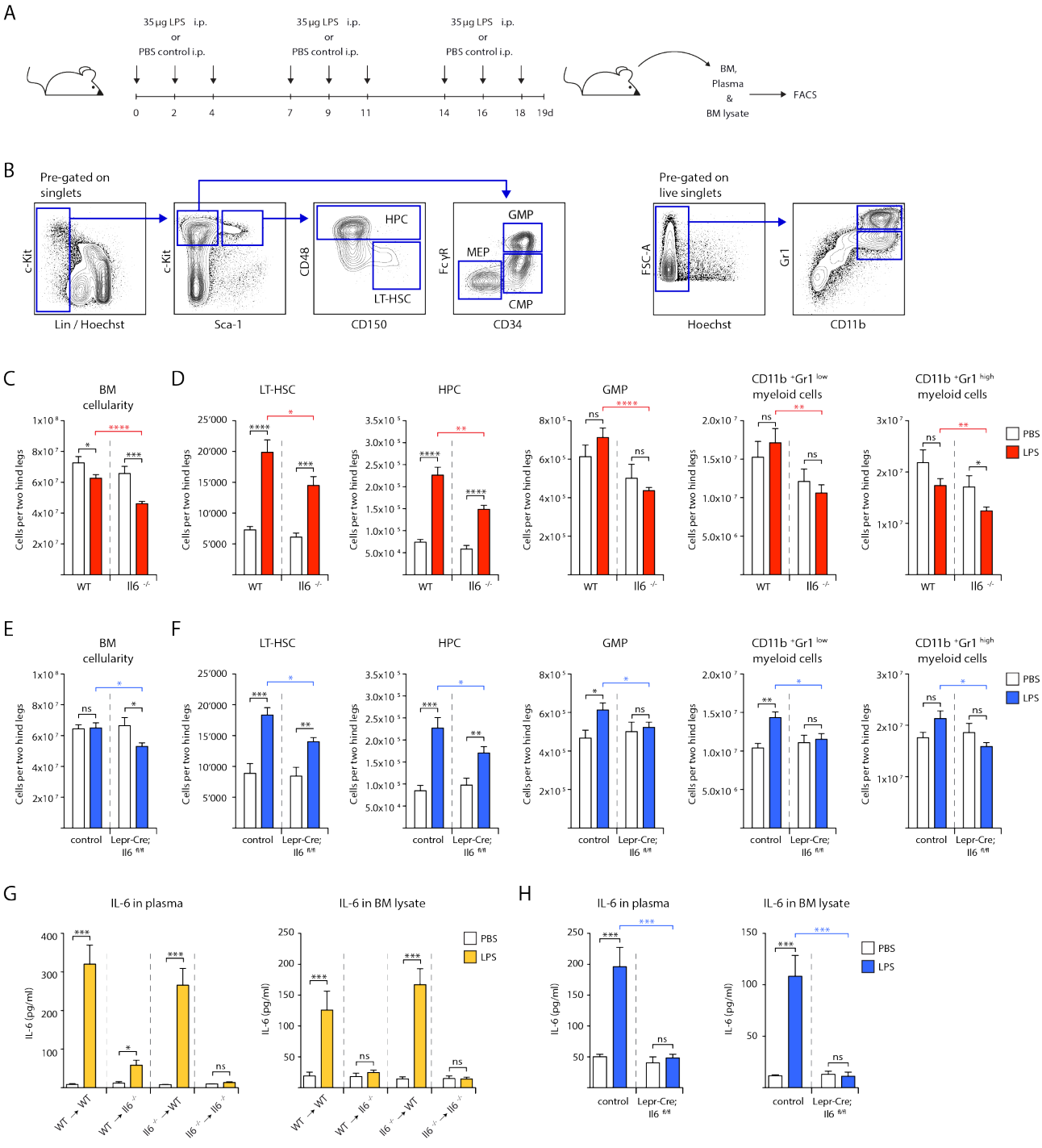
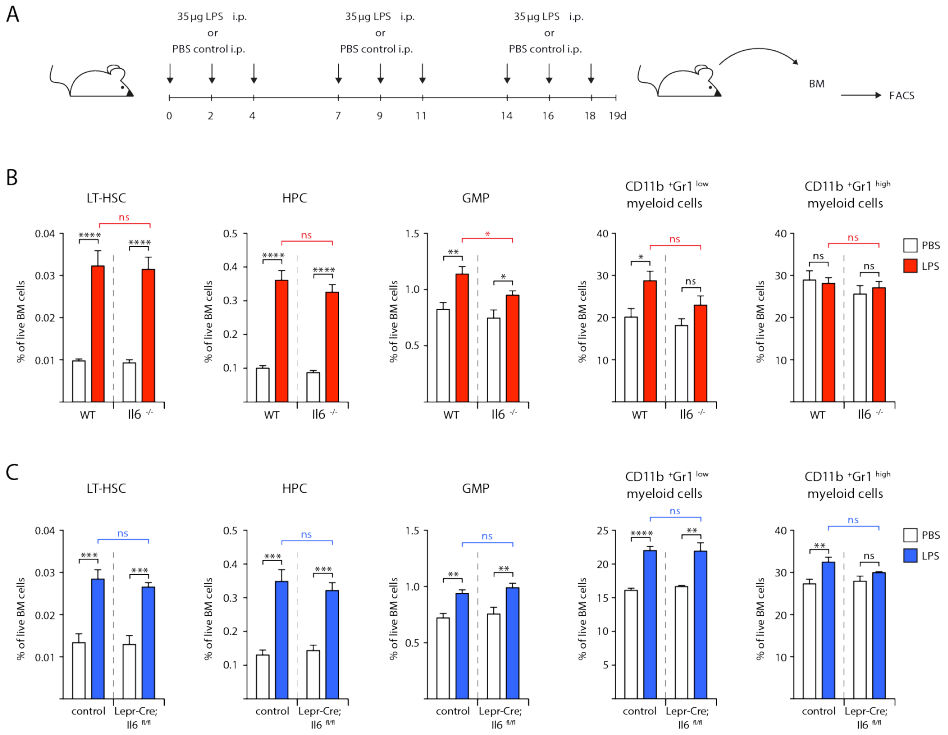


Figure 3. Hematopoietic response in BM of *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice upon chronic-repetitive LPS treatment.

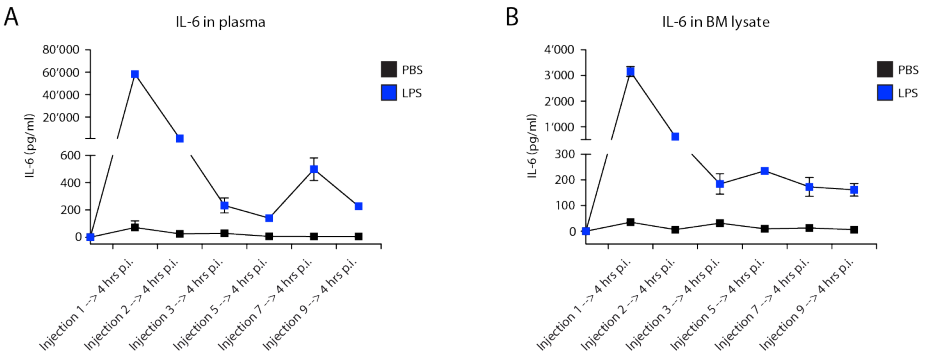
(A) Graphical scheme depicting experimental outline for modeling chronic inflammation by repetitive LPS injections over a period of 3 weeks followed by analysis of different hematopoietic cell types as well as measurement of IL-6 levels in plasma and BM lysates. This schedule is applicable to experimental data depicted in panels C – H. **(B)** Representative FACS profile

showing gating strategy for the identification of hematopoietic stem and progenitor cells (LT-HSC and HPC), myeloerythroid progenitors (CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor) as well as immature ($CD11b^{+}Gr1^{low}$) and mature granulocytic cells ($CD11b^{+}Gr1^{high}$) in PBS- and LPS-treated *Il6*$^{-/-}$, *Lepr-Cre;Il6*$^{fl/fl}$, and respective control mice. **(C)** BM cellularity and **(D)** absolute numbers per hind leg of immunophenotypically-defined LT-HSCs, HPCs, GMPs, $CD11b^{+}Gr1^{high}$ and $CD11b^{+}Gr1^{low}$ cells in LPS (red bars) or PBS (white bars) treated WT and *Il6*$^{-/-}$ mice according to the treatment schedule depicted under Fig. 3A. **(E)** BM cellularity and **(F)** absolute numbers of immunophenotypically-defined LT-HSCs, HPCs, GMPs, $CD11b^{+}Gr1^{high}$ and $CD11b^{+}Gr1^{low}$ cells in LPS (blue bars) or PBS (white bars) treated control and *Lepr-Cre;Il6*$^{fl/fl}$ mice according to the treatment schedule depicted under Fig. 3A. **(G)** IL-6 protein levels as measured by ELISA in plasma and BM lysates of *Il6*$^{-/-}$ BM reciprocal chimeric mice following PBS (white bars) or LPS (yellow bars) injection according to the experimental scheme shown in Fig. 3A. **(H)** IL-6 protein levels as measured by ELISA in plasma and BM lysates of *Lepr-Cre;Il6*$^{fl/fl}$ mice following PBS (white bars) or LPS (blue bars) injection according to the experimental scheme shown in Fig. 3A. (ns, non-significant; *, p=0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).



Supplemental Figure 4. Hematopoietic response in BM of *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice upon chronic-repetitive LPS treatment.

(A) Graphical scheme depicting experimental outline for modeling chronic inflammation by repetitive LPS injections over a period of 3 weeks followed by analysis of different hematopoietic cell types by FACS. This schedule is applicable to experimental data depicted in panels B and C. **(B)** Percentages of immunophenotypically-defined LT-HSCs, HPCs, GMPs, CD11b^{+}Gr1^{high} and CD11b^{+}Gr1^{low} cells in the BM of LPS (red bars) or PBS (white bars) treated WT and *Il6*$^{-/-}$ mice according to the treatment schedule depicted under Fig. S4A. **(C)** Percentages of immunophenotypically-defined LT-HSCs, HPCs, GMPs, CD11b^{+}Gr1^{high} and CD11b^{+}Gr1^{low} cells in the BM of LPS (blue bars) or PBS (white bars) treated control and *Lepr-Cre;Il6*$^{fl/fl}$ mice according to the treatment schedule depicted under Fig. S4A. Data from three independent experiments are shown.



Supplemental Figure 5. Longitudinal analysis of systemic and BM IL-6 levels during chronic-repetitive LPS administration.

(A) and **(B)** systemic and local BM IL-6 levels in WT mice during chronic-repetitive LPS stimulation (blue square) as compared to PBS-injected (black squares) control mice. Mice received the indicated number of LPS or PBS-injections (1, 2, 3, 5, 7, or 9 injections) over a maximum period of 3 weeks and were sacrificed 4 hours after the last injection. Data from three independent experiments are shown.

IL-6 is required to increase the number of functional myeloid CFUs during chronic-repetitive LPS-induced inflammation *in vivo* and is able to directly stimulate enhanced myeloid CFU activity *in vitro*

Having demonstrated that CAR cell-derived IL-6 plays a critical role in appropriately regulating quantities of immunophenotypically defined HSPC and myeloid cell populations during chronic-repetitive LPS-induced inflammation, we set out to also functionally evaluate these findings as there is only a weak correlation between HSPC immunophenotype and HSPC functionality, especially under inflammatory conditions known to alter expression of cell surface molecules commonly used to immunophenotypically define HSPC populations [133]. To this end, WT and *Il6*$^{-/-}$ mice received a chronic-repetitive high-dose LPS treatment of 9 injections during 3 weeks. Twenty-four hours after the last LPS injection, mice were euthanized, whole BM cells were isolated and subjected to a fully cytokine-supplemented (incl. IL-6) colony-forming unit (CFU) assay which represents the gold-standard for detection of functional HSPCs *in vitro* (Figure 4A). Not surprisingly, there was a substantial and highly significant increase in total colony formation primarily due to increased numbers of granulocytic colonies (CFU-G) in LPS-treated WT mice as compared to PBS-injected WT mice (Figure 4B). In stark contrast to LPS-treated WT mice, chronic-repetitive LPS treatment of *Il6*$^{-/-}$ mice failed to increase colony formation and also led to a significant decrease of total CFUs, not only resulting from a decrease in CFU-G but rather a more general decline in CFU activity (Figure 4B). Of note, the fact that IL-6 was contained in the cytokine cocktail used in our CFU assay demonstrates that *in vitro* IL-6 supplementation does not suffice to rescue the hematopoietic phenotype *in vitro* resulting from *in vivo* IL-6 deficiency. Importantly, when we conducted the same experiment as described above in *Lepr-Cre;Il6*$^{fl/fl}$ and control mice, we observed an identical response (Figure 4C) corroborating that CAR cell-derived IL6 is indeed primarily responsible for increasing hematopoietic output from HSPCs during emergency hematopoiesis induced by chronic-repetitive LPS treatment.

Next, we addressed the question as to whether CAR cell-derived IL-6 could act directly on HSPCs or via indirect mechanisms. First, we assessed cell surface expression of the ligand-binding subunit of the Interleukin-6 receptor, i.e. IL-6Rα (CD126) on HSPC and myeloid cell populations by flow-cytometry. We could observe detectable CD126 expression on all HSPC cell

populations except for megakaryocyte-erythrocyte progenitors (MEPs), however at very different intensities (Figure 4D). Interestingly, with the exception of MEPs, CD126 expression as measured by mean fluorescence intensity (MFI) gradually increased within the HSPC populations with lowest CD126 expression found in $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{+}CD48^{-}$ LT-HSCs and highest expression detected in $Lin^{-}Sca\text{-}1^{-}c\text{-}kit^{+}CD34^{+}Fc\gamma R^{+}$ GMPs (Figure 4D). CD126 expression could also be robustly detected in more differentiated myeloid cells ($CD11b^{+}Gr1l^{ow}$ and $CD11bGr1^{high}$) albeit at lower levels than in GMPs. Strikingly, when we evaluated the expression of gp130 (CD130) which acts as the signal-transducing subunit of the IL-6R, we observed an inverse expression pattern with highest CD130 expression in LT-HSCs and lowest expression in GMPs (Figure 4E). These results demonstrate that HSPCs including immunophenotypically defined LT-HSCs by virtue of their CD126 / CD130 expression possess the necessary prerequisites to functionally respond to IL-6 via IL-6R *cis*- and *trans*-signaling. Moreover, the observation that GMPs exhibit highest CD126 expression may suggest that GMPs represent the major IL-6 responsive HSPC population by *cis*-signaling whereas LT-HSCs may be particularly sensitive for IL-6 *trans*-signaling.

In order to functionally test whether HSPCs could directly respond to IL-6 we performed serial re-plating CFU assays with c-kit-enriched HSPCs isolated from BM of steady-state WT mice cultured *in vitro* in the presence or absence of IL-6 but in otherwise fully cytokine-supplemented methylcellulose medium (Figure 4F). We observed that the number of possible serial re-platings was independent of the presence or absence of IL-6 as both HSPCs cultured *in vitro* with or without IL-6 were incapable of forming colonies beyond the first re-plating (Figure 4F). However, and of note, HSPCs cultured in the presence of IL-6 were able to generate significantly more colonies both during the initial culture period as well as following the first re-plating when equal numbers of cells harvested from the initial cultures were re-plated (Figure 4F). These data strongly suggest that IL-6 could directly act on HSPCs, and that IL-6 signaling prolongs *in vitro* hematopoietic progenitor (HPC) maintenance.

Altogether, our results clearly demonstrate that CAR cell-derived IL-6, most likely via direct *cis*- or *trans*-signaling in HSPC populations, is critical to sustain emergency hematopoiesis during chronic-repetitive LPS-induced inflammation, as IL-6 deficiency in CAR cells leads to a failure to

adequately increase absolute numbers and functionality of HSPCs resulting in reduced BM cellularity, reduced BM myeloid CFUs, and decreased numbers of BM granulocytes.

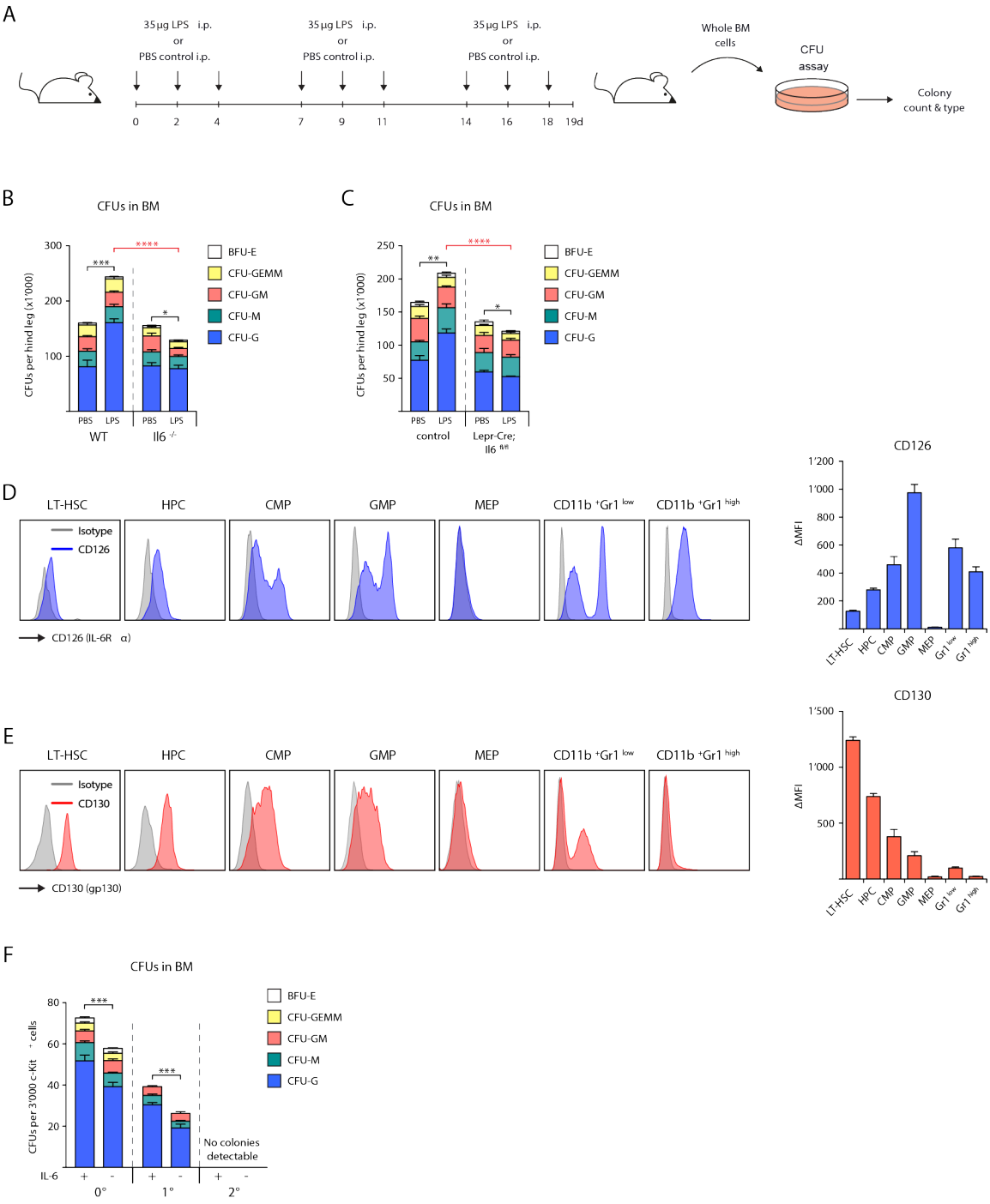


Figure 4. CFUs in BM of *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice upon chronic-repetitive LPS treatment and evidence for direct IL-6 action on HSPCs.

(A) Experimental outline illustrating chronic-repetitive LPS treatment of *Il6*$^{-/-}$, *Lepr-Cre;Il6*$^{fl/fl}$ and control mice followed by collection of whole BM cells which were subjected to colony-forming unit (CFU) assays. This schedule is applicable to experimental data depicted in panels B and C. **(B)** and **(C)** CFU activity shown as absolute CFU number per 1 hind leg in the BM of *Il6*$^{-/-}$, *Lepr-*

Cre;Il6$^{fl/fl}$ and respective control mice after chronic-repetitive *in vivo* LPS treatment according to Fig. 4A. **(D)** Left panel shows a representative flow-cytometric analysis for CD126 (IL-6R alpha chain, blue histograms) expression in LT-HSC, HPC, CMP, GMP, MEP, CD11b^{+}Gr1^{high} and CD11b^{+}Gr1^{low} cells in steady-state WT mice as compared to appropriate isotype controls (grey histograms). Right panel shows the delta mean fluorescent intensities (MFI) as calculation of MFI testor – MFI isotype control from 3 independent experiments. **(E)** Left panel shows a representative flow-cytometric analysis for CD130 (gp130, red histograms) expression in LT-HSC, HPC, CMP, GMP, MEP, CD11b^{+}Gr1^{high} and CD11b^{+}Gr1^{low} cells in steady-state WT mice as compared to appropriate isotype controls (grey histograms). Right panel shows the delta mean fluorescent intensities (MFI) calculated as described above. Results from 3 independent experiments are shown. **(F)** CFU activity in c-Kit-enriched BM cells isolated from steady-state WT mice and cultured in the presence or absence of IL-6 but in otherwise fully cytokine-supplemented methylcellulose medium (initial plating 0°). After 7 days in culture, colonies were scored, cells harvested, 3'000 cells were re-plated (1° re-plating) and grown under the same conditions (i.e. +/- IL-6) for another 7 days. Upon the second re-plating (2°) no colony growth could be observed. (CFU-G, CFU granulocyte; CFU-M, CFU macrophage; CFU-GM, CFU granulocyte/macrophage; CFU-GEMM, CFU granulocyte/ erythrocyte/ macrophage/ megakaryocyte; BFU-E, burst-forming unit erythrocyte). (ns, non-significant; *, p=0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

Systemic and local BM IL-6 levels during LPS-induced chronic-repetitive inflammation depend on *Tlr4* expression by non-hematopoietic cells

Our above-described data reveals a regulatory pathway that translates inflammatory signals derived from LPS stimulation into secretion of IL-6 from BM-resident CAR cells, and IL-6, in turn, then acts on HSPCs to sustain emergency hematopoiesis during chronic-repetitive inflammation. However, it remained to be determined whether sensing of LPS occurs directly in CAR cells followed by IL-6 secretion or whether LPS sensing is accomplished in another cell type that, in turn, stimulates IL-6 secretion from CAR cells via an intermediate signal.

To address this question, first, we determined expression of all relevant murine Toll-like receptors (*Tlr1-9*) in CAR cells by RT-qPCR. We also isolated, and then pooled conventional dendritic cells (cDCs) as well as plasmacytoid dendritic cells (pDCs) - thereafter referred to as DCs – to serve as a control for expression analyses since DCs are known to robustly express *Tlr* genes [32] (Figure 5A). We observed surprisingly high expression of a variety of *Tlr* genes in CARs at similar levels compared to DCs, in which TLR biology has been studied most extensively (Figure 5B). Importantly, among the most highly expressed *Tlr* genes in CAR cells was *Tlr4* – the cognate receptor for LPS thereby demonstrating that CAR cells are equipped with the molecular machinery to sense and respond to LPS. In order to test whether CAR cells are directly involved in LPS sensing *in vivo*, we faced the problem that available methodologies do not allow to specifically ablate *Tlr4* in CAR cells *in vivo*. Therefore, we generated reciprocal BM chimeric mice with *Tlr4* expression compartmentalized to either hematopoietic (WT ⇒ $Tlr4^{-/-}$) or non-hematopoietic cells ($Tlr4^{-/-}$ ⇒ WT) and respective control mice (WT ⇒ WT and $Tlr4^{-/-}$ ⇒ $Tlr4^{-/-}$). We subjected these mice to either a single high-dose LPS injection or chronic-repetitive high-dose treatment and measured IL-6 levels in plasma and BM lysates by ELISA (Figure 5C). While LPS sensing by both hematopoietic and non-hematopoietic cells equally contributes to local BM IL-6 levels following a single high-dose LPS injection (Figure 5D right panel), systemic IL-6 levels largely depend on *Tlr4* expression by non-hematopoietic cells irrespective of the duration of LPS treatment (Figure 5D right panel and Figure 5F left panel). Strikingly however, upon chronic-repetitive LPS stimulation, local BM IL-6 levels are maintained exclusively by non-hematopoietic cells (Figure 5F right panel). Although we cannot definitively rule out a contribution of other non-hematopoietic cell types in

LPS-sensing leading to IL-6 secretion from CAR cells, our above-described data demonstrating strong *Tlr4* expression in CAR cells in conjunction with our results from systemic and local BM IL-6 levels in *Tlr4*$^{-/-}$ BM chimeric mice suggest that CAR cells may indeed directly sense LPS *in vivo*.

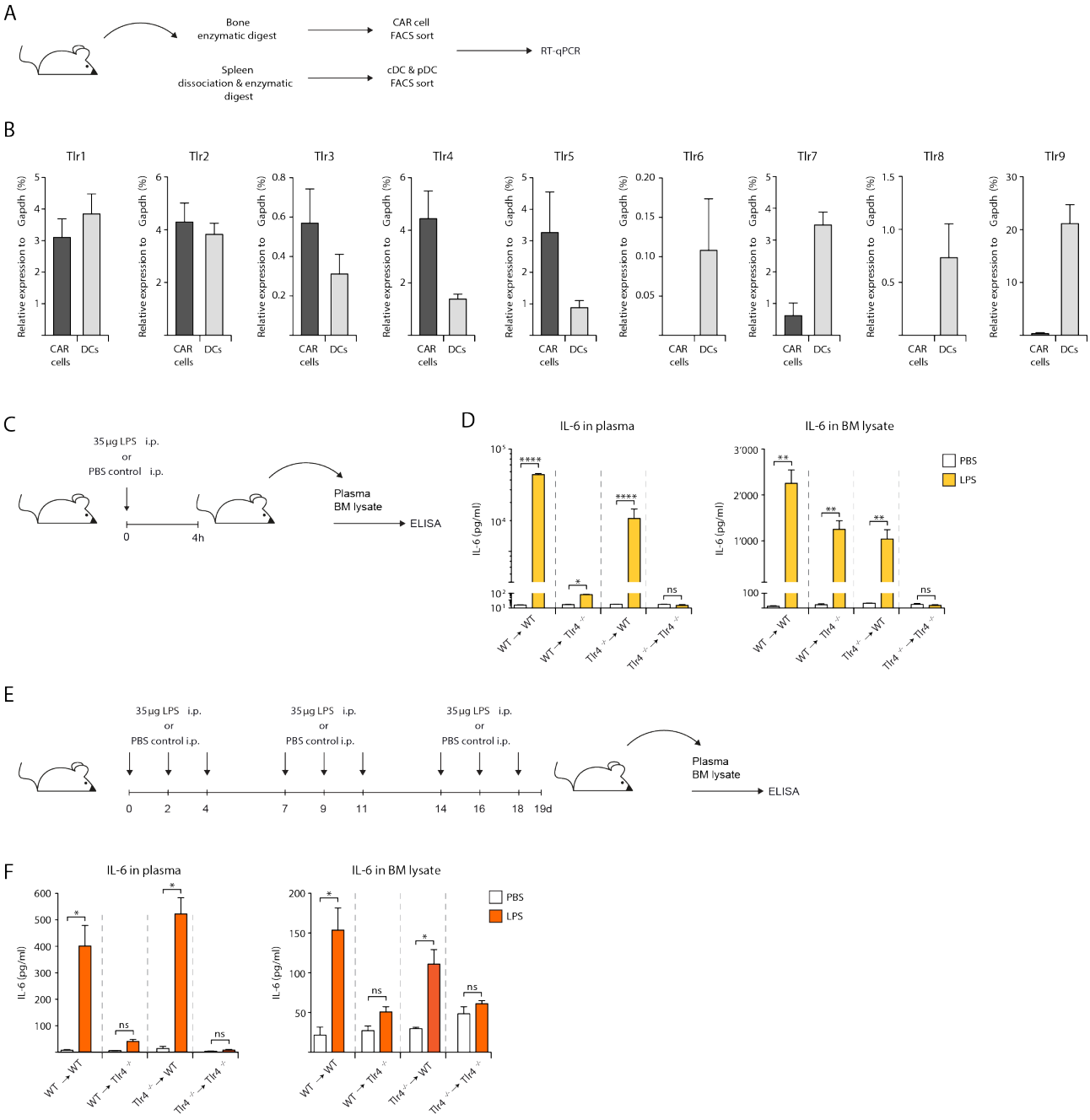


Figure 5. Toll-like receptor expression by non-hematopoietic stromal cell populations and IL-6 levels in reciprocal *Tlr4*$^{-/-}$ mice upon chronic-repetitive LPS treatment.

(A) Experimental outline depicting isolation of CAR cells from BM as well as conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) from spleen of steady-state WT mice for the assessment of *Tlr* expression as shown in Fig. 5B. **(B)** *Tlr1-9* expression as assessed by RT-qPCR in CAR cells (black bars) and DCs (pooled cDCs and pDCs, grey bars) isolated from steady-state WT mice. Expression values were normalized to housekeeping gene *Gapdh*. **(C)** Experimental

scheme showing induction of LPS-induced inflammation by a single LPS injection. This schedule is applicable to experimental data depicted in panel D. **(D)** IL-6 plasma and BM lysate levels in *Tlr4*$^{-/-}$ BM reciprocal chimeric mice upon a LPS-induced inflammation (yellow bars) in comparison to PBS-injected control mice (white bars). **(E)** Graphical scheme depicting experimental outline for chronic-repetitive LPS-induced inflammation. This schedule is applicable to experimental data depicted in panel F. **(F)** IL-6 plasma and BM lysate levels in *Tlr4*$^{-/-}$ BM reciprocal chimeric mice upon a LPS-induced inflammation (orange bars) in comparison to PBS-injected control mice (white bars). (ns, non-significant; *, p=0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

Discussion

In this study, we intended to get a deeper insight into how the non-hematopoietic BM microenvironment, that is known to be essential for proper functionality of steady-state hematopoiesis, may also be involved in regulating inflammation-driven emergency hematopoiesis. We hypothesized that, first, non-hematopoietic BM cells would be responsive towards infectious / inflammatory signals, and that second, inflammation-induced changes in non-hematopoietic BM cells would also reflect essential growth factor requirements of HSPCs during inflammation-driven emergency hematopoiesis. Therefore, we performed expression analyses for a selection of twelve prototypic hematopoietic growth factors and cytokines on different prospectively isolated non-hematopoietic cells from mice that were stimulated *in vivo* with LPS or poly(I:C) to mimic bacterial or viral infection, respectively. We identified *Il6* to be significantly, highly and relatively specifically up-regulated in CAR cells upon LPS but not poly(I:C) stimulation. Additional extensive experimentation comprising *Il6* expression analyses in whole tissue extracts from major solid organs and total bone, reciprocal *Il6*$^{-/-}$ BM chimeric mice, and conditional ablation of *Il6* in CAR cells using *Lepr-Cre;Il6*$^{fl/fl}$ mice yielded results that clearly demonstrated that CAR cells are the primary source of IL-6 during LPS-induced inflammation. These results seem surprising given the prevailing notion that inflammatory cytokines are mostly secreted from *bona fide* immune cells such as monocytes, macrophages and T cells. However, it has been known for a long time that both hematopoietic as well as non-hematopoietic cell types are capable of constitutive and inducible production of hematopoietic cytokines but these data are mostly derived from *in vitro* studies of cell lines or *in vitro* cultured primary cells [134-137]. Importantly, the *in vivo* contribution of various cell types to cytokine abundance under specific inflammatory settings is largely unknown and the here presented data demonstrates the need for proper *in vivo* experimentation when studying biological consequences of temporospatial and contex-dependent cytokine availabilities.

Another caveat might be the assumed low frequency of non-hematopoietic BM cells including CAR cells in mouse BM [68, 138, 139] and that such a scarce cell population could hardly account for systemically measurable IL-6 levels. However, it needs to be noted that FACS-based quantification of non-hematopoietic BM cell numbers [120] is massively underestimating the

actual frequency of these cells in mouse BM when compared to more sophisticated imaging technologies [65, 77] indicating that cell isolation procedures performed prior to flow-cytometrical analyses lead to suboptimal cell recovery. Consequently, CAR cells are a much more abundant cell population than previously thought underscoring their importance as a major source of IL-6 during LPS-induced inflammation.

In longitudinal analyses we also observed remarkable differences between initial systemic and local BM IL-6 levels as compared to those measured after repeated LPS stimulation. A putative explanation for this observation is a phenomenon called endotoxin tolerance (ET) occurring during chronic gram-negative bacterial infection. ET is believed to be a protective mechanism to prevent excessive hyper-inflammation. During ET, monocytes and macrophages enter an unresponsive state as a consequence of repeated exposure to endotoxins (e.g. LPS) and hence show an inability to respond to further LPS challenges [140, 141]. Whether ET also occurs in CAR cells following chronic-repetitive LPS stimulation remains to be determined. Importantly, despite an initial high burst in IL-6 levels followed by a rapid decrease in maximum IL-6 levels during subsequent chronic-repetitive LPS stimulation, IL-6 remains continuously and significantly elevated, and local BM concentrations may even be higher where HSPCs respond towards CAR cell-derived IL-6.

Testing the functional hematopoietic consequences of IL-6 during short-term LPS-induced inflammation revealed that IL-6 deficiency does not cause a measurable deficit in the elicited emergency hematopoiesis response. This observation is in line with published data demonstrating that emergency granulopoiesis is mainly driven by G-CSF at least during the initial phase [4, 5, 56]. In stark contrast, when we prolonged LPS stimulation thereby mimicking chronic-repetitive inflammation / infection, *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice developed BM hypocellularity compared to IL-6-competent mice. Furthermore, FACS analyses demonstrated a moderate but highly significant impairment in the ability to increase absolute numbers of immunophenotypically-defined LT-HSCs and HPCs in *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice upon chronic-repetitive LPS stimulation. Moreover, IL-6 deficiency led to a severe quantitative deficit in myeloid specific progenitors (GMPs) and downstream granulocytic cells (CD11b^{+}Gr1$^{low/high}$). Importantly, these quantitative phenotypes also

resulted in a severe functional defect in generating CFUs in the BM of LPS-treated *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice.

How do these results compare to published hematopoietic functions of IL-6? Although it has been known for over 20 years that IL-6 promotes hematopoiesis [112],two recent papers have reported on the role of IL-6 during inflammation-induced emergency hematopoiesis using state-of-the-art methodologies [39, 57].

Using single-cell proteomics Zhao et al. found that HSPCs are able to sense TLR agonists *in vitro* leading to the secretion of inflammatory cytokines including IL-6 which, in turn, could act in an autocrine / paracrine manner to stimulate enhanced HSPC proliferation and myeloid differentiation. While this interesting finding is likely to be biologically meaningful, it was challenging to rigorously test its functionally importance. Zhao et al. therefore devised an elegant experiment demonstrating that, under very specific experimental conditions of BM transplantation in a severely leukopenic host devoid of endogenous HSPCs, transplanted donor WT HSPCs were more efficient to generate myeloid offspring as opposed to *Il6*$^{-/-}$ donor HSPCs. Of note, our results presented here unambiguously demonstrated that hematopoietic cell-derived IL-6 is dispensable for emergency hematopoiesis during chronic-repetitive LPS stimulation but clearly showed that CAR cells are the major source of IL-6. This obvious discrepancy can be reconciled by the different experimental approaches reflecting fundamentally different clinical settings. The findings by Zhao et al. suggest that HSPC-derived IL-6 may be functionally relevant during the initial phase of hematopoietic recovery following myeloablative hematopoietic stem cell transplantation when the BM microenvironment is dysfunctional as a consequence of the conditioning regimen, and HSPCs may be able to compensate for growth factor deficiencies by the described autocrine / paracrine loop. However, our results obtained from a naturally-occurring setting of chronic infection / inflammation unequivocally demonstrates that the relative contribution of hematopoietic cells incl. HSPCs to IL-6 levels is negligible but depends primarily on non-hematopoietic CAR cells.

Furthermore, Schuerch et al. have recently reported a very interesting finding that, during viral infection, cytotoxic T cell-derived IFN stimulates IL-6 release from MSCs which, in turn, promotes monopoiesis. Of note, MSCs are distinct to CAR cells and thus, the findings by Schuerch et al. obtained from studies of acute viral infection as opposed to the here studied model setting of

chronic-repetitive bacterial infection do not contradict our data. On the contrary, our results in conjunction with findings by Schuerch et al. clearly demonstrate the versatility, flexibility and most importantly, context-dependency of growth factor provision by the BM microenvironment and should stimulate further research in this area.

We also addressed the question as to whether CAR cell-derived IL-6 acts directly or indirectly on HSPCs thereby sustaining emergency hematopoiesis during chronic-repetitive inflammation. To this end, we assessed expression of both CD126 (i.e. IL-6Rα) representing the ligand-binding subunit of the IL-6R as well as CD130 (i.e. gp130) representing the signal-transducing subunit. We observed detectable CD126 and CD130 expression at varying levels in HSPC and more differentiated myeloid cell populations with the exception of MEPs. Notably, we found an inverse pattern of differential expression between CD126 and CD130. While LT-HSCs express relatively low levels of CD126 but high levels of CD130, the opposite pattern was observed in GMPs. It needs to be noted that IL-6 signaling can either occur via membrane-bound IL-6R (*cis*-signaling) or through soluble IL-6Rα (*trans*-signaling). The latter dimerizes with membrane-bound gp130 triggering downstream signaling in a cell that does not endogenously express CD126 thereby increasing the target range of putative IL-6 responsive cell types. It is tempting to speculate that the differential expression of CD126 and CD130 in LT-HSCs versus GMPs has a functional relevance but further studies are required to test this hypothesis.

Finally, we set out to address the question as to whether CAR cells are directly sensing LPS followed by IL-6 secretion or whether LPS-sensing is accomplished in another cell type that, in turn, delivers an intermediate signal to CAR cells leading to IL-6 secretion. Due to current technical limitations, our data cannot provide a definitive answer to this question as this would require the ability to specifically delete *Tlr4* in CAR cells. However, our data strongly suggests that CAR cells are indeed directly sensing LPS as indicated by their high expression of *Tlr4*, the cognate receptor for LPS, and, based on to the observation that – especially during chronic-repetitive LPS-stimulation – systemic and local BM IL-6 levels depend on *Tlr4* expression by non-hematopoietic cells as determined in reciprocal BM *Tlr4*$^{-/-}$ chimeric mice.

In summary, using gene expression analyses in prospectively isolated non-hematopoietic BM cell populations in combination with several mouse models we here show that CAR cells are

the primary source of systemic and local BM IL-6 following LPS stimulation. Most importantly, while IL-6 is dispensable during the initial phase of LPS-induced emergency hematopoiesis, it is essential to sustain emergency hematopoiesis during chronic-repetitive inflammation. Our findings highlight the previously underappreciated and emerging role of the BM microenvironment as active participant in innate immune responses and critical regulator of demand-adapted hematopoiesis during infection and inflammation.

Author Contributions

R.C.G. and S.B. devised, performed and analyzed experiments, and wrote the manuscript; L.V.K. and A.H.performed experiments; C.N.B. and W.D.H. devised experiments and discussed data; and M.G.M. directed the study and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Results III

This is an article about the involvement of endothelial cells in the translation of pathogen signals into emergency granulopoiesis. I contributed to this article by assisting in the conduction of experiments such as flow cytometry cell sorting, qPCR and Elisa analysis.

HEMATOPOIESIS AND STEM CELLS

Endothelial cells translate pathogen signals into G-CSF–driven emergency granulopoiesis

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Key Points

- ECs express *Tlr4* and *Myd88* and, after in vivo LPS or *E coli* stimulation, are the prime sources of G-CSF.
- ECs are sensors of systemically spread pathogens and subsequent drivers of BM emergency granulopoiesis.

Systemic bacterial infection induces a hematopoietic response program termed "emergency granulopoiesis" that is characterized by increased de novo bone marrow (BM) neutrophil production. How loss of local immune control and bacterial dissemination is sensed and subsequently translated into the switch from steady-state to emergency granulopoiesis is, however, unknown. Using tissue-specific myeloid differentiation primary response gene 88 (*Myd88*)-deficient mice and in vivo lipopolysaccharide (LPS) administration to model severe bacterial infection, we here show that endothelial cells (ECs) but not hematopoietic cells, hepatocytes, pericytes, or BM stromal cells, are essential cells for this process. Indeed, ECs from multiple tissues including BM express high levels of *Tlr4* and *Myd88* and are the primary source of granulocyte colony-stimulating factor (G-CSF), the key granulopoietic cytokine, after LPS challenge or infection with *Escherichia coli*. EC-intrinsic MYD88 signaling and subsequent G-CSF production by ECs is required for myeloid progenitor lineage skewing toward granulocyte-macrophage progenitors, increased colony-forming unit granulocyte activity in BM, and accelerated BM neutrophil generation after LPS stimulation. Thus, ECs catalyze the detection of systemic infection into demand-adapted granulopoiesis. (*Blood*. 2014;124(9):1393-1403)

Introduction

Granulocytes are generated from upstream bone marrow (BM) precursors under the control of various myeloid cytokines, most importantly granulocyte colony-stimulating factor (G-CSF).[1] Among granulocytes, neutrophils are the dominant cell type in BM, peripheral blood (PB), and tissues in the steady state and during bacterial infection. Neutrophils serve as key first line of defense cells of the innate immune system.[2] To exert their critical function, neutrophils are recruited to respective sites of infection.[2] Although some neutrophils are able to reenter the vasculature,[3-5] the majority of cells are consumed and undergo cell death in the inflamed tissue as a consequence of launching their antimicrobial defense mechanisms.[2] In locally poorly controlled and consequently systemically spread microbial infection, neutrophils are in high demand and need to be regenerated in large numbers. Hence, because of their already short half-life in the steady state, ranging from a few hours to a few days,[6] during severe systemic infection, steady-state granulopoiesis is switched to "emergency granulopoiesis" (ie, massively enhanced de novo neutrophil production in BM).[7,8] Thus, besides efficient neutrophil recruitment to sites of infection and microbicidal neutrophil effector functions,[2] the third critical component of a protective innate immune response is the ability of the hematopoietic system to launch the emergency granulopoiesis program.

A prerequisite for the initiation of emergency granulopoiesis is sensing of pathogen dissemination. Pattern-recognition receptors such as Toll-like receptors (TLRs) recognize respective conserved pathogen-associated molecular patterns as diverse as proteins, polysaccharides, and nucleic acids.[9] TLRs can be found on the cell surface as well as within endosomal compartments, and TLR signal transduction occurs via adaptor molecules such as MYD88 and TRIF, leading to transcription of genes involved in the host response toward invading pathogens.[10] TLR expression and function has been best characterized in mature immune effector cells.[9] In addition, accumulating evidence has revealed that nonhematopoietic cells such as bladder epithelial cells,[11] mesenchymal stromal cells,[12,13] and endothelial cells (ECs)[14,15] also express TLRs, thereby critically contributing to innate immune responses. Furthermore, it has been demonstrated that hematopoietic stem and progenitor cells (HSPCs) express some TLRs and directly respond to pathogen-associated molecular patterns with increased myelopoiesis and directed migration to inflamed sites.[16-22] However, we have recently shown that direct pathogen sensing by HSPCs does not play an essential role in the immediate lipopolysaccharide (LPS)-induced emergency granulopoiesis response. By contrast, TLR4 agonist sensing by nonhematopoietic cells followed by granulopoietic growth factor release, primarily G-CSF, is the main route for the initiation of emergency granulopoiesis.[23]

We address here the fundamental question of which cell type out of the vast plethora of putative nonhematopoietic cells and tissues is

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the key sensor of systemically spread pathogens, specifically LPS and *Escherichia coli*, and how this sensing is translated into emergency granulopoiesis.

Methods

Mice

C57BL/6J (CD45.2$^+$), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1$^+$), *Myd88*$^{-/-}$, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, *LysM-Cre*, *Nes-Cre*, *Pdgfrb-Cre*, *Alb-Cre*, *Tie2-Cre*, *Myd88*$^{fl/fl}$ (B6.129P2(SJL)-*Myd88*^{tm1Defr}/J), and *loxP-GFP* (B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAGZsGreen1)Hze}$/J) mice were used in this study. All animals were maintained at the University Hospital Zurich animal facility and treated in accordance with guidelines of the Swiss Federal Veterinary Office. Experiments and procedures were approved by the Veterinäramt des Kantons, Zurich, Switzerland.

LPS injections, *E coli* infection, and G-CSF injections

Mice received 2 intraperitoneal injections with 35 μg ultrapure LPS from *E coli 0111:B4* (InvivoGen) 48 hours apart and were analyzed 24 hours after the second injection. For some experiments, mice were administered 20 μg LPS 3 times (at 0, 12, and 20 hours) and were analyzed at time point 24 hours. Alternatively, mice were intraperitoneally (IP)-injected with 0.5 to 4.5 × 10^8 *E coli* and analyzed 48 hours later. Some mice were IP-injected with 250 μg/kg body weight human G-CSF (Filgrastim; Amgen) 6 times in a 12-hour interval and analyzed as LPS-injected mice.

Flow cytometry

The following antibodies (all from eBiosciences, unless otherwise stated) were used to assess mature BM and PB cell populations: anti-CD11b (M1/70), anti-Gr1 (RB6-8C5), anti-Ly6-G (1A8; Biolegend), anti-Ly6-C (HK1.4), anti-B220 (RA3-6B2), and anti-CD3ε (145-2C11). For fluorescence-activated cell sorter (FACS) analysis of HSPCs, the following antibodies were used: anti-CD3ε (145-2C11), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-B220 (RA3-6B2), anti-CD19 (MB19-1), anti-CD11b (M1/70), anti-Gr1 (RB6-8C5), anti-Ter119 (Ter-119), anti-IL7Rα (A7R34), anti-cKit (2B8), anti-Sca1 (D7), anti-CD34 (RAM34), anti-FcgR (93), and anti-NK1.1 (PK136; Becton Dickinson).

ECs were stained with anti-CD45 (30-F11), anti-TER119 (Ter-119), and anti-CD31 (390). In some experiments, anti-VE-cadherin (BV13), anti-Sca1 (E13-161.7; BD Biosciences), anti-VCAM1 (429), and anti-CD105 (MJ7/18) antibodies were used.

Classical and plasmacytoid dendritic cells were stained using anti-CD3ε (145-2C11), anti-CD19 (MB19-1), anti-NK1.1 (PK136; Becton Dickinson), anti-CD45RA (14.8; BD Biosciences), anti-CD11c (N418), and anti-MHCII (M5/114.15.2).

Hoechst33342 (Life Technologies) was used to exclude dead cells. Cells were analyzed or sorted on a FACS Canto II or FACS Aria III flow cytometer (BD Biosciences), respectively. Data were analyzed by FlowJo (Tree Star) software.

BrdU incorporation assay

Mice were injected with phosphate-buffered saline (PBS) or LPS according to the scheme depicted in Figure 5A. Twelve hours before analysis, mice received 1 single IP injection of 2 mg 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich). In addition, BrdU at a final concentration of 0.8 mg/mL as well as 5% glucose were added to the drinking water. Cells were isolated and further processed according to the manufacturer's protocol (BrdU Flow Kit; BD Pharmingen).

Colony-forming unit (CFU) assay

After in vivo PBS or LPS stimulation according to the scheme depicted in Figure 1A, 1 × 10^4 red blood cell–depleted whole BM cells were plated in methylcellulose (Methocult M3234; StemCell Technologies) mixed with Iscove's modified Dulbecco's medium (30% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol) with the following factors added: mIL3 (10 ng/mL), hIL6 (10 ng/mL), hIL11 (10 ng/mL), mFLT3-Ligand (10 ng/mL), mSCF (10 ng/mL), mGM-CSF (10 ng/mL), huTPO (50 ng/mL), and huEPO (4 U/mL) (all R&D Systems). Colonies were scored after 8 days of in vitro culture.

Quantitative reverse-transcription PCR

ECs were sorted directly into lysis buffer belonging to RNeasy Micro Kit (QIAGEN). Total RNA extraction was carried out according to the manufacturer's protocol and subsequently subjected to reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR green reagent (Applied Biosystems) and samples were run on a 7500 FAST real-time PCR thermal cycler (Applied Biosystems). *Actb* was used to normalize the RNA content of samples.

Enzyme-linked immunosorbent assay

PB was obtained from mice postmortem by cardiac puncture using heparinized syringes and centrifuged to obtain plasma. G-CSF plasma levels were measured using Quantikine ELISA kits according to the manufacturer's instructions (R&D Systems).

Equations and statistical analyses

Significance of differences was analyzed using Student *t* test. A difference between experimental groups was considered significant when the *P* value was $<.05$. All statistical analyses were calculated with Prism software (GraphPad Software, version 5.01).

Results

LPS-induced emergency granulopoiesis requires intact MYD88-mediated TLR signaling in nonhematopoietic cells

To examine whether MYD88 expression within hematopoietic or nonhematopoietic cells is required for LPS-induced emergency granulopoiesis, we generated BM chimeric mice with MYD88 expression restricted to either the hematopoietic (wild-type [WT]→*Myd88*$^{-/-}$) or the nonhematopoietic cellular compartment (*Myd88*$^{-/-}$→WT) as well as respective control mice (WT→WT and *Myd88*$^{-/-}$→*Myd88*$^{-/-}$) (Figure 1A). We IP-injected chimeric mice with high doses of LPS to mimic severe systemic infection (Figure 1A) and analyzed typical surrogate hallmarks of emergency granulopoiesis. Although absolute leukocyte counts did not change significantly in PB upon LPS stimulation (data not shown), the frequency and absolute numbers of PB CD11b$^+$ cells significantly increased in WT→WT mice (Figure 1B). This response was completely absent in *Myd88*$^{-/-}$→*Myd88*$^{-/-}$ mice, indicating that LPS-induced elevation in PB CD11b$^+$ cells depends on MYD88 signaling. Strikingly, although WT→*Myd88*$^{-/-}$ mice were also completely nonresponsive toward LPS, *Myd88*$^{-/-}$→WT mice showed a normal LPS response (Figure 1B). Next, we analyzed the BM of chimeric mice after LPS treatment. We found that total BM cell numbers are significantly decreased upon LPS stimulation in WT→WT mice (Figure 1C). The reduction of BM cellularity is mediated in a MYD88-dependent manner because *Myd88*$^{-/-}$→*Myd88*$^{-/-}$ mice did not show this response. This depends on MYD88 expression within nonhematopoietic cells because *Myd88*$^{-/-}$→WT mice responded normally but WT→*Myd88*$^{-/-}$ mice lacked this response (Figure 1C). A characteristic feature of emergency granulopoiesis is a significant, relative, and absolute decrease in BM CD11b$^+$Gr1^{high} mature neutrophils (Figure 1D-E). This response is paralleled by a significant relative increase in BM CD11b$^+$Gr1^{low} immature neutrophils[23-25] and

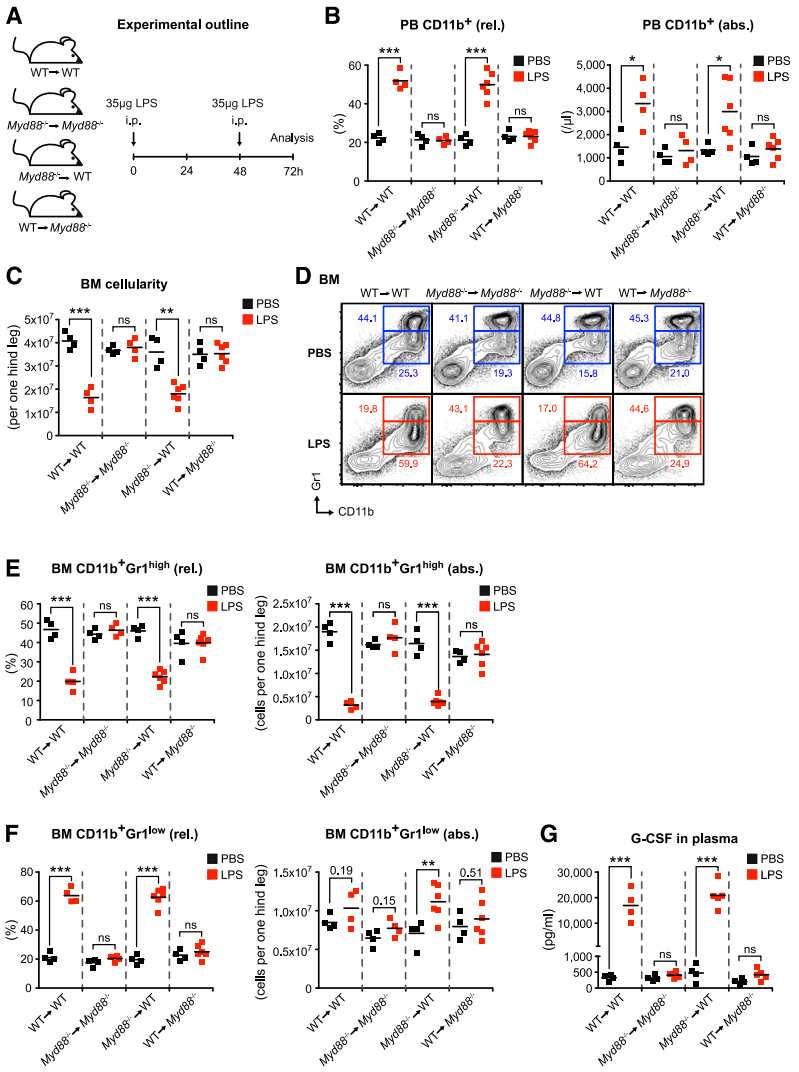

Figure 1. LPS-induced emergency granulopoiesis requires intact MYD88-mediated TLR signaling in nonhematopoietic cells. (A) Graphical scheme depicting experimental outline to induce LPS-induced emergency granulopoiesis in mice with MYD88 expression restricted to hematopoietic (WT→*Myd88*$^{-/-}$) or nonhematopoietic (*Myd88*$^{-/-}$→WT) cells, and respective control mice (WT→WT and *Myd88*$^{-/-}$→*Myd88*$^{-/-}$). (B) Frequency and absolute number of PB CD11b^{+} cells, and (C) BM cellularity after LPS stimulation in BM chimeric and control mice. (D) Representative FACS profile showing characteristic changes in BM CD11b^{+}Gr1^{high} mature and BM CD11b^{+} Gr1^{low} immature neutrophils after LPS stimulation. (E) Frequencies and absolute numbers of BM CD11b^{+}Gr1^{high} mature, (F) BM CD11b^{+}Gr1^{low} immature neutrophils, and (G) plasma G-CSF levels in reciprocal *Myd88*$^{-/-}$ BM chimeras upon systemic LPS injection. Black squares, PBS-injected mice; red squares, LPS-injected mice; rel., relative; abs., absolute. Data from 2 independent experiments are shown. Two-tailed Student *t* tests were used to assess statistical significance (*$P < .05$, **$P < .01$, ***$P < .001$; ns, nonsignificant).

a trend toward an absolute increase in BM CD11b^{+}Gr1^{low} immature neutrophils. These reciprocal changes in BM CD11b^{+}Gr1^{high} mature neutrophils and BM CD11b^{+}Gr1^{low} immature neutrophils after LPS injection require intact MYD88 signaling in nonhematopoietic cells (Figure 1D-F).

Although various cytokines such as granulocyte-monocyte colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor, interleukin-6, and G-CSF have the capacity to stimulate both steady-state as well as emergency granulopoiesis, none of these cytokines is absolutely essential.[1,26-29] However, G-CSF plays an outstanding role in these processes as demonstrated in studies of *G-csf*$^{-/-}$ and *G-csfr*$^{-/-}$ mice showing hematopoietic defects in steady state, and most importantly, upon infection.[1,30,31] Notably, administration of recombinant G-CSF is routinely used to treat iatrogenic[32] and congenital[33] neutropenia. Furthermore, G-CSF alone is sufficient to induce a response indistinguishable from LPS-induced emergency granulopoiesis.[23,34] We therefore assessed G-CSF plasma levels in BM chimeric mice after LPS stimulation. We found highly significant increases in plasma G-CSF levels in WT→WT and *Myd88*$^{-/-}$→WT mice (Figure 1G). By contrast, *Myd88*$^{-/-}$→*Myd88*$^{-/-}$ and WT→*Myd88*$^{-/-}$ mice did not show elevated plasma G-CSF levels. Thus, LPS-triggered G-CSF release requires intact *Myd88* within nonhematopoietic cells.

To corroborate our findings on the importance of nonhematopoietic cells for the initiation of emergency granulopoiesis and to further rule out a role for tissue-resident macrophages for this process, we generated *LysM-Cre;Myd88*$^{fl/fl}$ mice with a *Myd88* deficiency restricted to myeloid cells including macrophages.[35] In line with the results on hematopoietic chimeric animals, we observed that

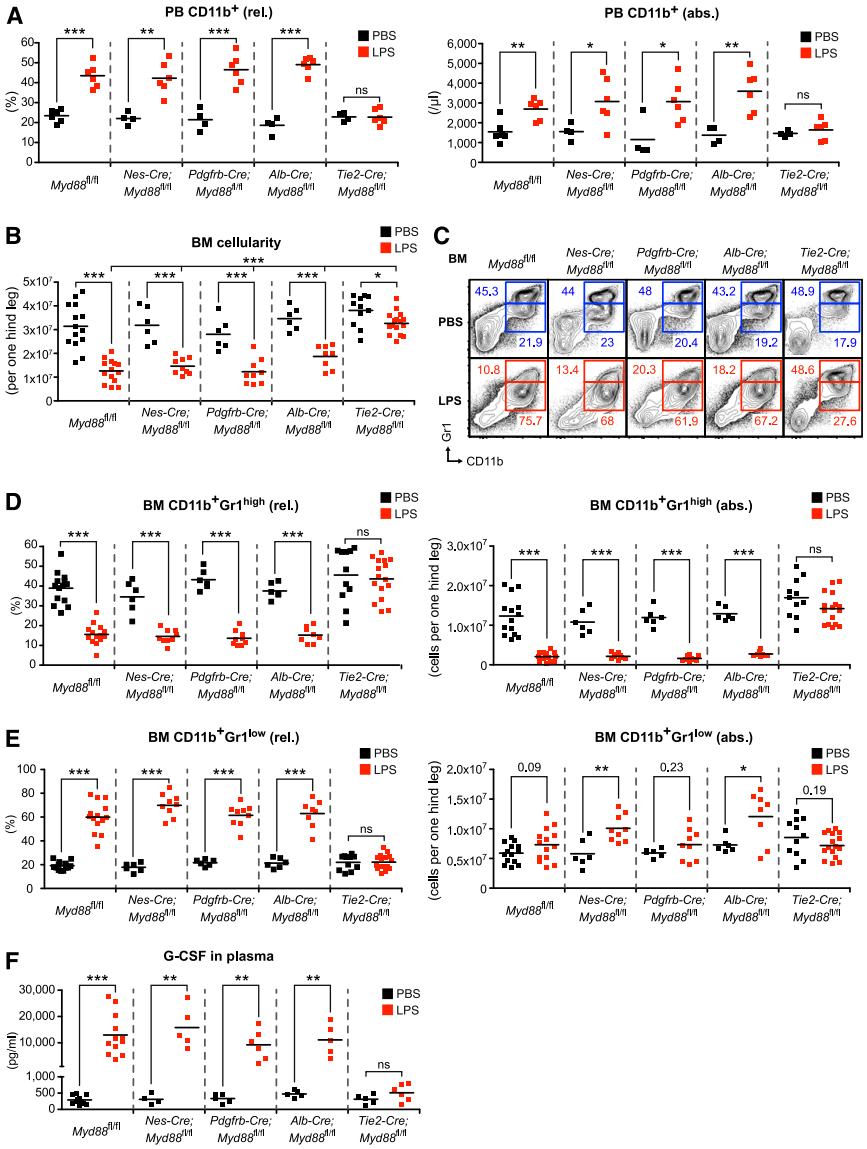

Figure 2. LPS-induced emergency granulopoiesis is abrogated in *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (A) Frequency and absolute number of PB CD11b^{+} cells after LPS stimulation in control *Myd88*$^{fl/fl}$, and experimental *Nes-Cre;Myd88*$^{fl/fl}$, *Pdgfrb-Cre;Myd88*$^{fl/fl}$, *Alb-Cre;Myd88*$^{fl/fl}$, and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (B) BM cellularity after LPS stimulation in control *Myd88*$^{fl/fl}$, and experimental *Nes-Cre;Myd88*$^{fl/fl}$, *Pdgfrb-Cre;Myd88*$^{fl/fl}$, *Alb-Cre;Myd88*$^{fl/fl}$, and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (C) Representative FACS profile showing characteristic LPS-induced changes in BM CD11b^{+}Gr1^{high} mature and BM CD11b^{+}Gr1^{low} immature neutrophils in control *Myd88*$^{fl/fl}$, and experimental *Nes-Cre;Myd88*$^{fl/fl}$, *Pdgfrb-Cre;Myd88*$^{fl/fl}$, *Alb-Cre;Myd88*$^{fl/fl}$, 0and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (D) Frequencies and absolute numbers of BM CD11b^{+}Gr1^{high} mature and (E) BM CD11b^{+}Gr1^{low} immature neutrophils, and (F) plasma G-CSF levels in the different sets of tissue-specific *Myd88*$^{-/-}$ mice after systemic LPS injection. Black squares, PBS-injected mice; red squares, LPS-injected mice; rel., relative; abs., absolute. Data from at least 3 independent experiments are shown. Two-tailed Student *t* tests were used to assess statistical significance (*$P < .05$, **$P < .01$, ***$P < .001$).

LysM-Cre;Myd88$^{fl/fl}$ mice are fully capable to launch emergency granulopoiesis (supplemental Figure 1, available on the *Blood* Web site).

Altogether, our results on MYD88 and previous data on TLR4[23] unambiguously show that LPS-induced emergency granulopoiesis is initiated through TLR agonist sensing and MYD88-dependent signaling by nonhematopoietic cells.

LPS-induced emergency granulopoiesis is abrogated in *Tie2-Cre;Myd88*$^{fl/fl}$ mice

To unravel the identity of the nonhematopoietic cell type critical for emergency granulopoiesis, we used *Cre-loxP* recombination technology to delete *Myd88* from various candidate nonhematopoietic cell

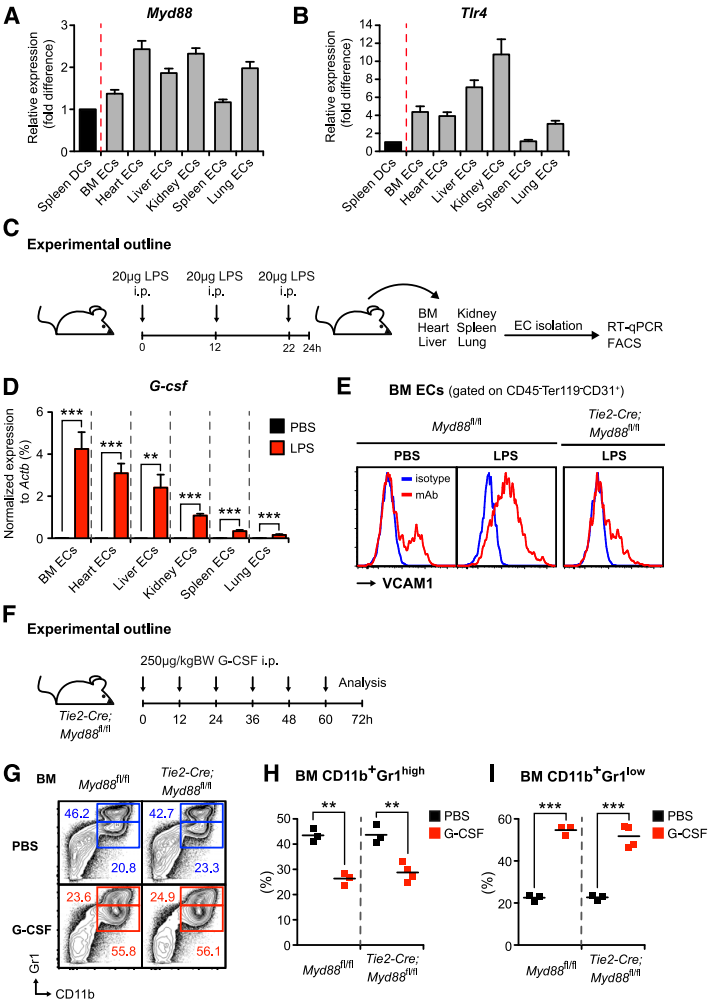

Figure 3. Endothelial cells from various organs express high levels of *Myd88* and *Tlr4*, respond to LPS challenge with strong upregulation of VCAM-1 and *G-csf* in vivo, and exogenous G-CSF administration rescues emergency granulopoiesis in *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (A) *Myd88* and (B) *Tlr4* expression were assessed by quantitative reverse-transcription PCR in CD45$^-$Ter119$^-$CD31$^+$ECs isolated from BM, heart, liver, kidney, spleen, and lung (gray bars) compared with spleen DCs (black bars), ie, pooled classical dendritic cells (CD3ε$^-$CD19$^-$NK1.1$^-$CD11c^{high}CD45RA$^-$MHCII$^+$) and plasmacytoid dendritic cells (CD3ε$^-$CD19$^-$NK1.1$^-$CD11c$^+$CD45RA$^+$MHCII^{high}). All cells were isolated from steady-state mice. (C) Graphical scheme depicting experimental outline to assess in vivo LPS responsiveness of ECs that were flow-cytometrically sorted from BM, heart, liver, kidney, spleen, and lung of PBS- and LPS-injected mice, respectively. (D) Comparative *G-csf* transcript levels normalized to *Actb* in ECs from BM, heart, liver, kidney, spleen, and lung of LPS-injected (red bars) vs PBS-injected (black bars) wild-type mice. (E) Representative FACS analysis depicting cell-surface expression of VCAM1 (red lines) on BM ECs in steady state as well as LPS-injected control *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. Isotype control shown as blue line. (F) Graphical scheme showing experimental outline to assess G-CSF effects in vivo. (G) Representative FACS profile showing characteristic G-CSF–induced changes in BM CD11b$^+$Gr1^{high} mature and BM CD11b$^+$Gr1^{low} immature neutrophils in control *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (H) Frequencies of BM CD11b$^+$Gr1^{high} mature and (I) BM CD11b$^+$Gr1^{low} immature neutrophils in PBS- and G-CSF–injected control *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. All data represent mean ± standard deviation from 2 or 3 independent experiments. Two-tailed Student *t* tests were used to assess statistical significance (**$P < .01$, ***$P < .001$).

populations to study their relative contribution to pathogen sensing (supplemental Table 1). To achieve *Myd88* deletion in cells of the BM microenvironment (*Nestin*$^+$ mesenchymal stem cells and perivascular stromal cells), we used *Nes-Cre*[36-38] and *Pdgfrb-Cre*[39] mice. Cells belonging to the vasculature (ECs and pericytes) were targeted using *Tie2-Cre*[40] and *Pdgfrb-Cre*[39] mice, respectively. We also employed *Alb-Cre*[41] mice to target hepatocytes as a candidate parenchymal organ cell type. First, we assessed the LPS response in PB of control and tissue-specific *Myd88*$^{-/-}$ mice. We observed that the frequency and absolute numbers of PB CD11b$^+$ cells significantly increased in LPS-injected *Myd88*$^{fl/fl}$ control, *Nes-Cre;Myd88*$^{fl/fl}$, *Pdgfrb-Cre;Myd88*$^{fl/fl}$, and *Alb-Cre;Myd88*$^{fl/fl}$ mice, whereas *Tie2-Cre;Myd88*$^{fl/fl}$ mice lacked the response (Figure 2A). The majority of PB CD11b$^+$ cells could be identified as neutrophils based on coexpression of Ly6-G, whereas PB CD11b$^+$Ly6-C^{high} inflammatory monocytes showed a relative decrease but overall stable absolute numbers (supplemental Figure 2).

Next, we investigated the BM response toward LPS. Not only did we observe a decrease in BM cellularity in LPS-injected *Myd88*$^{fl/fl}$ control, *Nes-Cre;Myd88*$^{fl/fl}$, *Pdgfrb-Cre;Myd88*$^{fl/fl}$, and *Alb-Cre;Myd88*$^{fl/fl}$ mice (Figure 2B), we also revealed a reduction in the frequency and absolute number of BM CD11b$^+$Gr1^{high} mature neutrophils (Figure 2C-D). Although the percentage of BM CD11b$^+$Gr1^{low} immature neutrophils was highly significantly increased, there was only a minor increase in absolute numbers that, likely because of the higher variation in BM cellularity, did not always yield statistical significance (Figure 2C,E). Strikingly, *Tie2-Cre;Myd88*$^{fl/fl}$ mice were nonresponsive toward systemic LPS injection, with the exception of a small but significant decrease in BM cellularity that was, however, significantly reduced in magnitude compared with the other groups of mice (Figure 2B-E). In line with these findings, G-CSF levels increased massively, up to 100-fold, in control and respective tissue-specific *Myd88*$^{-/-}$ mice with the exception of *Tie2-Cre;Myd88*$^{fl/fl}$ mice that lacked a significant rise in plasma G-CSF concentration (Figure 2F).

ECs express high levels of *Myd88* and *Tlr4* and respond to LPS challenge with strong upregulation of VCAM-1 and *G-csf* in vivo

Our findings suggested that ECs, which are targeted in *Tie2-Cre* mice, are the cells within the nonhematopoietic compartment responsible

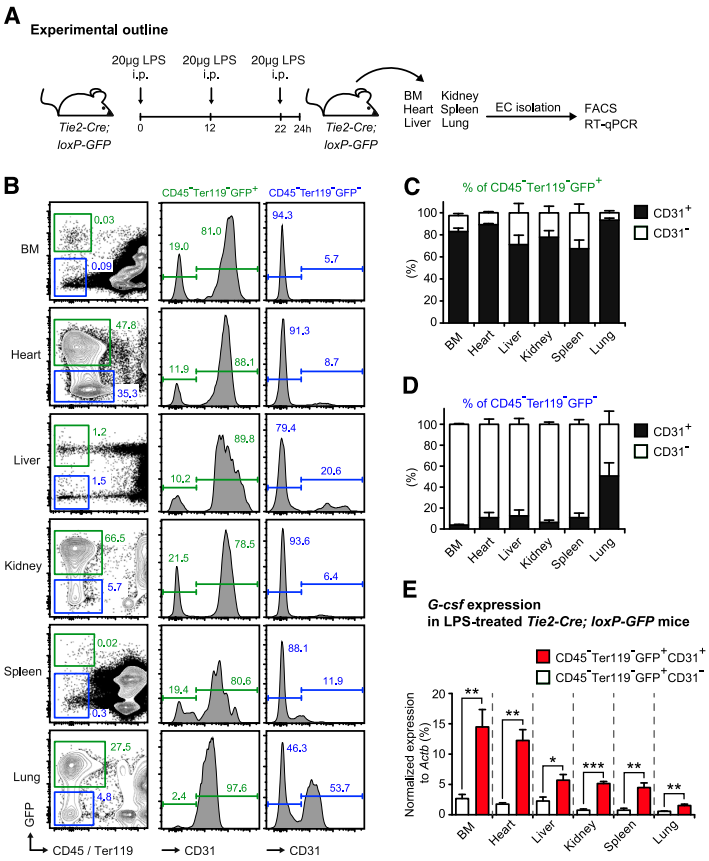

Figure 4. Endothelial cells are effectively targeted in *Tie2-Cre-loxP-GFP* reporter mice and are the main source of *G-csf* after in vivo LPS stimulation. (A) Graphical scheme depicting experimental outline to induce LPS-induced emergency granulopoiesis and to assess *G-csf* expression in sorted CD45$^-$Ter119$^-$GFP$^+$CD31$^+$ ECs and CD45$^-$Ter119$^-$GFP$^+$CD31$^-$ non-ECs isolated from different organs of *Tie2-Cre-loxP-GFP* reporter mice. (B) Representative FACS profile of GFP and CD31 expression within nonhematopoietic cells (CD45$^-$Ter119$^-$) isolated from BM, heart, liver, kidney, spleen, and lung of *Tie2-Cre-loxP-GFP* reporter mice. (C) Percentages of CD31$^+$ (black bars) and CD31$^-$ (white bars) cells within the CD45$^-$Ter119$^-$GFP$^+$ cell population in BM, heart, liver, kidney, spleen, and lung of *Tie2-Cre-loxP-GFP* reporter mice. (D) Percentages of CD31$^+$ (black bars) and CD31$^-$ (white bars) cells within the CD45$^-$Ter119$^-$GFP$^-$ cell population in BM, heart, liver, kidney, spleen, and lung of *Tie2-Cre-loxP-GFP* reporter mice. (E) Comparative *G-csf* transcript levels normalized to *Actb* in sorted CD45$^-$Ter119$^-$GFP$^+$CD31$^+$ ECs (red bars) vs CD45$^-$Ter119$^-$GFP$^+$CD31$^-$ non-ECs (white bars) isolated from BM, heart, liver, kidney, spleen, and lung of LPS-injected *Tie2-Cre-loxP-GFP* reporter mice. All data represent mean ± standard deviation from 2 independent experiments (n = 5 mice). Two-tailed Student *t* tests were used to assess statistical significance (*$P < .05$, **$P < .01$, ***$P < .001$).

for sensing LPS, translating this signal into G-CSF release, and consequently initiating emergency granulopoiesis.

To assess expression of *Tlr4* and *Myd88* in ECs, we isolated CD45$^-$Ter119$^-$CD31$^+$ ECs from BM, heart, lung, liver, spleen, and kidney and compared these with pooled spleen classical and plasmacytoid dendritic cells with defined TLR expression patterns.[42] No immunophenotypical differences were observed between ECs from different organs with respect to the endothelial markers VE-cadherin, CD105, CD34, and Sca1 (data not shown). *Myd88* and *Tlr4* were abundantly expressed in ECs at similar or higher levels compared with dendritic cells, ie, cell populations known for their TLR expression profile (Figure 3A-B).[20]

To analyze the functional relevance of *Tlr4* and *Myd88* expression in ECs, we injected mice with LPS or PBS (Figure 3C); isolated ECs from BM, heart, lung, liver, spleen, and kidney; and assessed *G-csf* transcripts by quantitative real-time PCR. Although in the PBS-injected steady-state mice, *G-csf* was only detectable at very low levels, LPS injection resulted in a strong upregulation of *G-csf* (up to several hundred-fold) in ECs from all organs analyzed (Figure 3D). Interestingly, *G-csf* induction was relatively highest in ECs from BM (ie, the primary site of hematopoiesis). However, it needs to be emphasized that this does not necessarily imply that absolute G-CSF amounts produced are also highest in this tissue. Transcription and protein production do not always correlate, and quantity of protein produced in a given organ will also depend on frequency of producing cells at site.

To test whether there might be heterogeneity in the responsiveness toward LPS within different sections of the vasculature, we analyzed the activation status of ECs upon systemic LPS stimulation by measuring VCAM1 cell-surface expression.[43] However, we did not detect differences between ECs isolated from different tissues and, importantly and in line with the observed nonresponsiveness, VCAM1 upregulation on ECs was abrogated in *Tie2-Cre;Myd88*$^{fl/fl}$ mice (Figure 3E and data not shown).

To confirm that disrupted MYD88 signaling in *Tie2-Cre;Myd88*$^{fl/fl}$ mice does not affect G-CSF signal transduction and consequently impairs emergency granulopoiesis, we injected *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice with recombinant human G-CSF (Figure 3F). In accordance with our previously published data on G-CSF effects in *Tlr4*$^{-/-}$ mice,[23] we observed that G-CSF alone without an additional inflammatory stimulus is sufficient to accurately mimic emergency granulopoiesis (Figure 3G-I and data not shown).

Given the possibility of insufficient tissue specificity of *Cre-loxP* technology, we generated *Tie2-Cre-loxP-GFP* reporter mice to study the effectiveness and specificity of EC targeting. To this end, we isolated nonhematopoietic cells from BM, heart, liver, kidney, spleen, and lung and analyzed both GFP and expression of the EC marker CD31 by FACS (Figure 4A-D). In accordance with previously published reports,[37] we found that the vast majority of ECs (approximately 90%) were efficaciously targeted in *Tie2-Cre-loxP-GFP* reporter mice. This pattern was robustly observed in all tissues analyzed, except

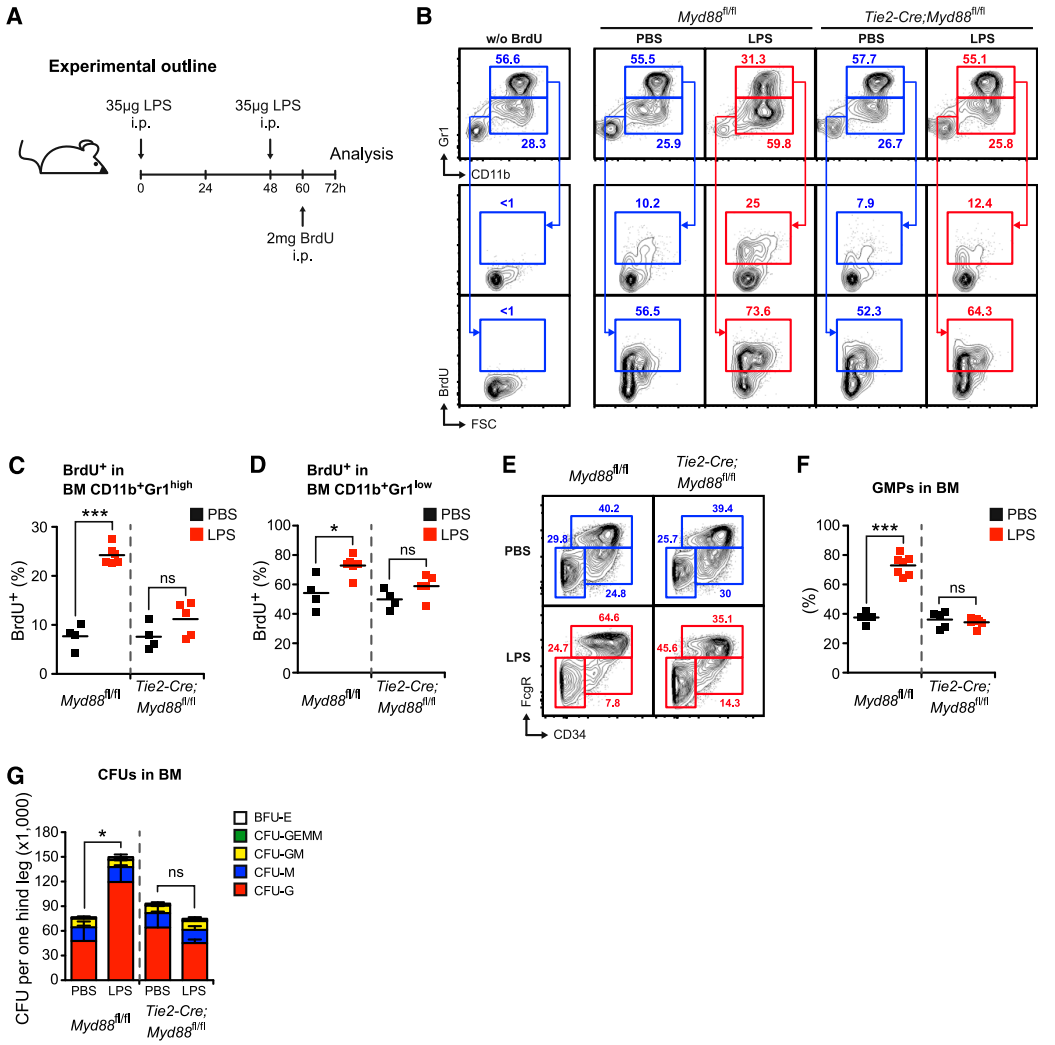

Figure 5. Endothelial cell–intrinsic MYD88 signaling is required to stimulate accelerated neutrophil production, myeloid progenitor lineage skewing toward granulocyte-macrophage progenitors (GMPs), and increased CFU-G activity in vivo. (A) Graphical scheme depicting experimental outline to induce LPS-induced emergency granulopoiesis and to assess BrdU incorporation. (B) Representative FACS profile showing BrdU incorporation in in BM CD11b$^+$Gr1^{high} mature and BM CD11b$^+$Gr1^{low} immature neutrophils in control *Myd88*$^{fl/fl}$, and *Tie2-Cre;Myd88*$^{fl/fl}$ mice during steady-state and LPS-induced emergency granulopoiesis. (C) Frequencies of BrdU$^+$ BM CD11b$^+$Gr1^{high} mature and (D) BrdU$^+$ BM CD11b$^+$Gr1^{low} immature neutrophils in PBS- or LPS-injected *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (E) Representative FACS profile showing myeloerythroid progenitors in control *Myd88*$^{fl/fl}$, and *Tie2-Cre;Myd88*$^{fl/fl}$ mice during steady-state and LPS-induced emergency granulopoiesis. (F) Frequencies of Lin$^-$cKit$^+$Sca1$^-$FcgR$^+$CD34$^+$ GMPs in PBS- or LPS-injected *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. (G) Absolute CFU numbers per 1 hind leg in control *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice during steady-state and LPS-induced emergency granulopoiesis (CFU-G, CFU granulocyte; CFU-M, CFU macrophage; CFU-GM, CFU granulocyte/macrophage; CFU-GEMM, CFU granulocyte/erythrocyte/macrophage/megakaryocyte; BFU-E, burst-forming unit erythrocyte). Black squares, PBS-injected mice; red squares, LPS-injected mice. Data from 2 independent experiments are shown. Two-tailed Student *t* tests were used to assess statistical significance (*$P < .05$, ***$P < .001$).

for the lung, where we found a higher fraction of CD31$^+$ cells within the CD45$^-$Ter119$^-$GFP$^-$ fraction (Figure 4B-D). Furthermore, we observed that about 20% (with some variations between the different tissues) of CD45$^-$Ter119$^-$GFP$^+$ cells in *Tie2-Cre-loxP-GFP* reporter mice do not express the EC marker CD31 and thus, presumably, are not ECs (Figure 4B-C).

To evaluate the role of these CD45$^-$Ter119$^-$GFP$^+$CD31$^-$ cells for the initiation of emergency granulopoiesis, we injected *Tie2-Cre-loxP-GFP* reporter mice with LPS, sorted the CD31$^+$ EC and CD31$^-$ non-EC fractions of the CD45$^-$Ter119$^-$GFP$^+$ nonhematopoietic compartment and compared *G-csf* expression by quantitative reverse-transcription PCR as a surrogate marker for the ability of the respective cell type to stimulate emergency granulopoiesis (Figure 4A). There was no difference in steady-state *G-csf* expression between both cell fractions (data not shown), whereas after LPS injection, *G-csf* expression in CD45$^-$Ter119$^-$GFP$^+$CD31$^+$ ECs was always

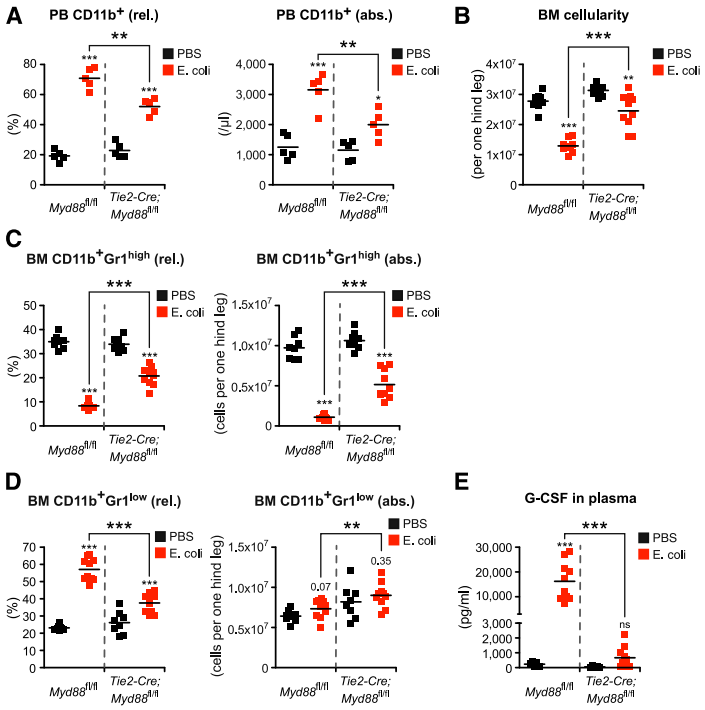

Figure 6. Endothelial cell–intrinsic MYD88 signaling is required to efficiently stimulate emergency granulopoiesis during systemic *E coli* infection. (A) Frequency and absolute number of PB CD11b$^+$ cells, (B) BM cellularity, (C) frequencies and absolute numbers of BM CD11b$^+$Gr1^{high} mature and (D) BM CD11b$^+$Gr1^{low} immature neutrophils, and (E) plasma G-CSF levels in *Myd88*$^{fl/fl}$ mice and *Tie2-Cre;Myd88*$^{fl/fl}$ mice in steady-state (black squares) and after infection with 4.5×10^8 *E coli* CFU (red squares) given IP. Data from 2 independent experiments are shown. rel., relative; abs., absolute. Two-tailed Student *t* test was used to assess statistical significance (**$P < .001$, ***$P < .001$).

significantly higher (2- to 5-fold) than in the CD45$^-$Ter119$^-$GFP$^+$CD31$^-$ non-EC fraction in all organs analyzed (Figure 4E). Thus, given the 4-fold higher numbers of CD45$^-$Ter119$^-$GFP$^+$CD31$^+$ ECs vs CD45$^-$Ter119$^-$GFP$^+$CD31$^-$ non-ECs and the 2- to 5-fold higher amount of *G-csf* expression in CD45$^-$Ter119$^-$GFP$^+$CD31$^+$ ECs vs CD45$^-$Ter119$^-$GFP$^+$CD31$^-$ non-ECs, we infer that most (>95%) of G-CSF derived from ECs and, importantly, *Tie2-Cre* mice, are a valid tool to assess ECs within the nonhematopoietic compartment.

Collectively, ECs are equipped with the functional machinery to detect LPS and are indeed the dominant G-CSF–producing cell population upon systemic LPS challenge, sufficient to induce emergency granulopoiesis.

EC-intrinsic MYD88 signaling is required to stimulate accelerated neutrophil production, myeloid progenitor lineage skewing toward granulocyte-macrophage progenitors, and increased CFU-G activity in vivo

Next, we assessed neutrophil generation in the BM of *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice in the steady state and during LPS-induced emergency granulopoiesis (Figure 5A). During 12 hours, approximately 10% and 50% of BM CD11b$^+$Gr1^{high} mature and BM CD11b$^+$Gr1^{low} immature neutrophils incorporated BrdU into newly synthesized DNA in PBS-injected control and *Tie2-Cre;Myd88*$^{fl/fl}$ mice, respectively (Figure 5B-D). After LPS injection, there was a significant increase in BrdU incorporation in *Myd88*$^{fl/fl}$ mice in both BM CD11b$^+$Gr1^{high} mature and BM CD11b$^+$Gr1^{low} immature neutrophils. However, this increase was absent in LPS-injected *Tie2-Cre;Myd88*$^{fl/fl}$ mice (Figure 5B-D). These results demonstrate that neutrophil turnover is significantly accelerated upon LPS stimulation, and that this response is dependent on EC-intrinsic MYD88 signaling.

We also analyzed the myeloid progenitor compartment in steady-state and LPS-injected *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. In accordance with previously published results,[44] we observed an increase in the percentage of Lin$^-$cKit$^+$Sca1$^-$FcgR$^+$CD34$^+$ granulocyte-macrophage progenitors (GMPs) upon LPS stimulation in *Myd88*$^{fl/fl}$ mice. This response was absent in *Tie2-Cre;Myd88*$^{fl/fl}$ mice (Figure 5E-F). These data demonstrate that LPS induces a lineage bias at the HPC level in favor of enhanced granulopoiesis.

Finally, we determined the CFU activity in BM from steady-state or LPS-injected *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice. After LPS injection, there was a significant increase in the overall number of CFUs in *Myd88*$^{fl/fl}$ mice. Notably, this increment was due to an increase in CFU-granulocyte (CFU-G) with no changes in the number of other CFU types. Importantly, this response was absent in LPS-treated *Tie2-Cre;Myd88*$^{fl/fl}$ mice (Figure 5G).

Taken together, these data unambiguously demonstrate that, besides its many known biological effects, including stimulation of hematopoietic stem cell division[45] and of neutrophil migration and recruitment to inflamed tissues,[14,15] LPS stimulates emergency granulopoiesis via MYD88 signaling in ECs.

EC-intrinsic MYD88 signaling is required to efficiently stimulate emergency granulopoiesis during systemic *E coli* infection

To evaluate whether MYD88 signaling in ECs is also important to initiating emergency granulopoiesis in a far more complex setting of infection with living bacteria, we performed experiments with

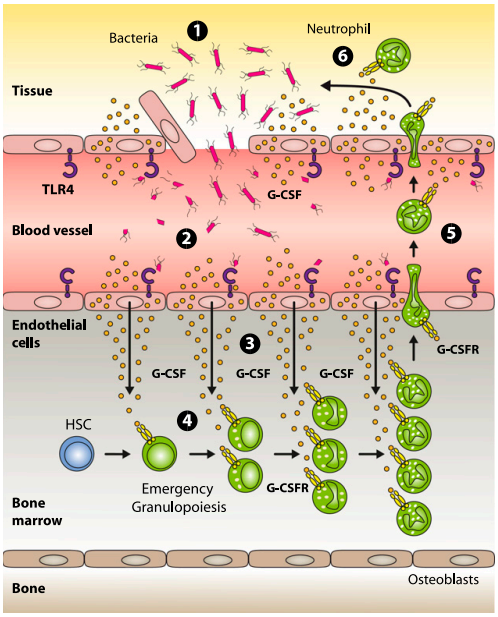

Figure 7. Model for pathogen sensing and subsequent translation into emergency granulopoiesis. (1) Gram-negative bacteria and/or their structural components that have gained access to the systemic circulation are recognized by TLR4-expressing endothelial cells (2), thereby indicating an emergency state. Upon TLR4/MYD88 signaling in endothelial cells, G-CSF is released in large quantities (3). In the bone marrow, endothelial cell–derived G-CSF acts on myeloid precursors expressing the G-CSF receptor resulting in enhanced generation, accelerated turnover, and increased neutrophil release from the bone marrow to the systemic circulation (4). These neutrophils are recruited to the site of infection (5) where they participate in clearing the pathogen (6).

E coli. First, we titrated the *E coli* dose necessary to elicit a similar quantitative response in WT mice as observed with the LPS dose used in prior experiments. We determined this dose to be 4.5×10^8 *E coli* CFU given IP per WT mouse (supplemental Figure 3A-E). In parallel, we also infected *Myd88*$^{-/-}$ mice. Although these mice showed some response to *E coli*, the responses were severely and highly significantly reduced compared with WT mice for the entire previously assessed readouts characteristic for emergency granulopoiesis (supplemental Figure 3A-E). Importantly, the minor MYD88-independent responses upon *E coli* infection are also independent of G-CSF because there was no significant rise in plasma G-CSF levels in *Myd88*$^{-/-}$ mice (supplemental Figure 3E).

Next, we infected control *Myd88*$^{fl/fl}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice with *E coli* (4.5×10^8 CFU per mouse). We observed the same pattern, with *Myd88*$^{fl/fl}$ mice showing the same response as WT mice, and *Tie2-Cre;Myd88*$^{fl/fl}$ mice showing a minor, in comparison with *Myd88*$^{fl/fl}$ mice significantly reduced response. This indicates that EC-intrinsic MYD88 signaling is required to induce full-blown emergency granulopoiesis also upon live *E coli* infection (Figure 6A-E). The minor response observed in *Tie2-Cre;Myd88*$^{fl/fl}$ mice in an infection with live bacteria might not be surprising because in this far more complex situation, in contrast to exclusive LPS-mediated stimulation, other pattern-recognition receptor agonists and additional tissue damage also might activate alternative, independent signaling pathways. Notably, however, plasma G-CSF levels did not rise above steady-state levels in *Tie2-Cre;Myd88*$^{fl/fl}$ mice, revealing ECs to be major sources of G-CSF also during infection with *E coli* (Figure 6E).

To test whether TLR adaptor TRIF might partially compensate for the severely diminished *E coli*–induced emergency granulopoiesis in *Myd88*$^{-/-}$ and *Tie2-Cre;Myd88*$^{fl/fl}$ mice, and to determine the relative contribution of each pathway to it, we infected WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ mice with 4.5×10^8 *E coli* CFU IP. We observed that both *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice show an equally reduced ability to launch emergency granulopoiesis compared with WT mice. By stark contrast, *Trif*$^{-/-}$ mice responded to *E coli* (supplemental Figure 4) with emergency granulopoiesis, as did WT mice. These data demonstrate that TRIF is dispensable for the initiation of emergency granulopoiesis.

In summary, our findings demonstrate that abrogated MYD88 signaling in ECs leads to severely defective emergency granulopoiesis upon *E coli* infection.

Discussion

We addressed here the fundamental question of which cell types act as primary sensors of bacterial dissemination during severe infection and consequently induce the switch from steady-state to demand-adapted emergency granulopoiesis.

Two general models have been proposed for how pathogen sensing and translation into emergency granulopoiesis may be achieved.[7,8] According to the concept of indirect hematopoietic activation, tissue-resident macrophages might serve in this critical function[46,47] because they indeed express pattern-recognition receptors, and, after respective stimulation, produce granulopoietic growth factors.[48-51] But data from stringent in vivo experimentation supporting this model are lacking. Alternatively, several recent studies suggested a model of direct hematopoietic activation based on the observation that mouse and human HSPCs express TLRs and that TLR agonist stimulation leads to enhanced myelopoieisis.[16-19,21] However, these studies used either in vitro[16] or very specific in vivo experiments such as adoptive HSPC transfer under the renal capsule,[18] direct injection of HSPCs into *Staphylococcus aureus*–infected wounds,[21] or pre-treatment with chemotherapeutic agents and ionizing radiation[22] to convey their respective proposed model. By contrast, we have demonstrated that pathogen sensing by both immature HSPCs and mature hematopoietic cells, including macrophages, is dispensable for the acute process of emergency granulopoiesis in 3 independent and complementary experimental approaches. First, in both hematopoietic *Tlr4*$^{-/-}$ chimeric mice[23] and hematopoietic *Myd88*$^{-/-}$ chimeric mice, emergency granulopoiesis is indistinguishable from that in WT mice. Of note, we and others could show that BM chimeric mice are an appropriate tool to broadly dissect biological effects contributed by either hematopoietic or nonhematopoietic tissues by confirming that tissue-resident macrophages, although they are able to locally self-renew in the steady state,[52] are replenished by donor-derived cells after lethal irradiation.[23,52] Second, liposomal clodronate–mediated macrophage-depleted WT mice launch undiminished emergency granulopoiesis upon LPS stimulation.[23] Third, as shown here, mice with a *Myd88*-deficiency in the myeloid compartment, most importantly macrophages (*LysM-Cre;Myd88*$^{fl/fl}$ mice), respond normally toward LPS with emergency granulopoiesis.

To determine the identity of the nonhematopoietic cell types required for the initiation of emergency granulopoiesis, we undertook an extensive genetic targeting approach using Cre recombinase–mediated tissue-specific *Myd88* ablation. Specifically, we targeted the nonhematopoietic BM microenvironment (*Nestin*$^{+}$ mesenchymal stem cells and perivascular stromal cells) using *Nes-Cre*[36-38] and *Pdgfrb-Cre*[39]

mice. These cells have been shown to be part of the hematopoiesis-supporting BM microenvironment.[53] Vascular cells (ECs and pericytes) were targeted using *Tie2-Cre*[37,38,40,54] and *Pdgfrb-Cre*[39] mice, respectively. In addition, we used *Alb-Cre*[41] mice to target hepatocytes as a candidate prototypic parenchymal cell type. Our data revealed that ECs express high amounts of *Tlr4* and *Myd88* and respond to systemic LPS stimulation or *E coli* infection with massive upregulation of the primary granulopoiesis-supporting growth factor G-CSF. Most importantly, mice with a *Myd88* deficiency in ECs do not respond with emergency granulopoiesis upon systemic LPS stimulation and have a severely defective response toward *E coli* infection. The *E coli*–driven MYD88- and G-CSF–independent minor responses in $Myd88^{-/-}$ and $Tie2\text{-}Cre;Myd88^{fl/fl}$ mice are likely triggered by activation of alternative, redundant pathways that evolved to secure this critical response. Of interest, a recent study showed that granulopoiesis after antibody-mediated neutrophil removal depends on TLR4/TRIF signaling.[55] We therefore studied whether a similar feedback regulatory mechanism via TLR4/TRIF might account for the residual emergency granulopoiesis observed in *E coli*–infected $Myd88^{-/-}$ and $Tie2\text{-}Cre;Myd88^{fl/fl}$ mice. However, we observed that TLR4 signal transduction via TRIF is dispensable for the induction of *E coli*–driven emergency granulopoiesis as $Trif^{-/-}$ mice show a response that is indistinguishable from those in WT mice.

Because congenital[33] or iatrogenic[32] forms of neutropenia are associated with a high incidence of lethal infectious complications, it seems very likely that the ability to induce emergency granulopoiesis per se has a live-saving role in massive infection. However, direct experimental evidence for this notion is lacking and future studies will need to dissect emergency granulopoiesis from neutrophil recruitment to inflamed tissues and neutrophil effector functions, which together ensure host survival during bacterial infection.

Given the general caveat of lack of absolute tissue specificity in the *Cre-loxP* system, we have analyzed *Tie2-Cre-loxP-GFP* reporter mice. In accordance with the literature,[37,38,40,54] *Tie2-Cre* mice are an excellent tool to genetically manipulate ECs, but, of note, there is also a small fraction of other (ie, non-EC and nonhematopoietic) cells targeted in *Tie2-Cre* mice. However, we have addressed this issue experimentally by analyzing the G-CSF response in non-ECs in these mice and could exclude a relevant contribution of these nonhematopoietic, non-EC cell populations to the overall emergency granulopoiesis response.

Interestingly, the versatile and critical function of endothelium is an emerging aspect in hematopoiesis research. It has recently been demonstrated that ECs are an important constituent of the BM microenvironment. Using a similar genetic tissue-specific deletion approach, ECs were shown to be major sources of stem cell factor[37] and CXCL12,[38,54] 2 molecules that are involved in HSPC maintenance and their BM retention, respectively. Our results now show that ECs are not only critical for the regulation of steady-state hematopoietic maintenance but are also essential for demand-adapted hematopoiesis in response to the paradigmatic gram-negative bacteria–derived compound LPS and the clinically relevant pathogen *E coli*. In conjunction with previously published data on the importance of TLR signaling in ECs for neutrophil recruitment to inflamed tissues,[14,15] our findings reveal a sophisticated degree of functional interplay between the vascular, the hematopoietic, and the immune systems, which have a common developmental root and might thus be understood as 1 functional organ.[56,57] Of note, our findings also confirm and extend prior landmark observations on the importance of nonhematopoietic cell-derived hematopoietic growth factors.[58,59] Although these seminal studies established a role for nonhematopoietic cells as a major source of hematopoietic growth factors, they, because of technical limitations at that time, were unable to identify the precise identity of this cell type that we now show to be ECs.

ECs are ideally positioned to mark the threshold of local vs systemic infection, and using ECs as safeguards seems a very plausible, danger-adapted defense strategy established in evolution. In summary, based on the data presented here, we propose a model (Figure 7) in which ECs are critical for pathogen sensing and subsequent induction of emergency granulopoiesis.

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Authorship

Contribution: S.B. devised, performed and analyzed experiments, and wrote the manuscript; R.C.G., R.R., J.B., and F.A. performed experiments; M.H. and M.K. devised experiments and discussed data; and M.G.M. directed the study and wrote the manuscript.

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Discussion & Outlook

It is well-established that the BM microenvironment plays an essential role in regulating the various aspects of steady-state hematopoiesis [60, 61, 64, 72-78, 80, 87-90]. Moreover, accumulating evidence suggests that non-hematopoietic BM cells also contribute to the hematopoietic system's adaptation to stress-induced also termed 'emergency hematopoiesis' [46, 56]. Therefore, we here addressed the question which regulatory signals are provided by non-hematopoietic cells of the BM microenvironment to govern the switch from steady-state to inflammation- and infection-driven hematopoiesis and to sustain the latter during an ongoing inflammatory process. To this end, we planned to mimic severe systemic bacterial or viral infection by applying LPS or poly(I:C), respectively, followed by flow-cytometrically sorting different non-hematopoietic BM cell types for subsequent RNA extraction and expression analysis.

As non-hematopoietic cell types in the BM comprise only around 0.1-0.8% of total cells, we undertook an extensive effort to establish and optimize a protocol for the prospective isolation of non-hematopoietic BM cell types. Using cell surface markers CD45 and Ter119 to exclude hematopoietic cells in combination with Sca-1, CD31, CD105 and CD140b, several non-hematopoietic BM cell types could be flow-cytometrically defined and robustly isolated from mouse BM: sinusoidal endothelial cells (SECs, $CD45^{-}Ter119^{-}CD31^{+}CD105^{hi}Sca\text{-}1^{int}$), arteriolar endothelial cells (AECs, $CD45^{-}Ter119^{-}CD31^{+}CD105^{int}Sca\text{-}1^{hi}$), mesenchymal stromal cells (MSCs, $CD45^{-}Ter119^{-}CD31^{-}Sca1^{+}CD140b^{+}$), and CXCL12-abundant reticular cells (CAR cells, $CD45^{-}Ter119^{-}CD31^{-}Sca1^{-}CD140b^{+}$). As a pilot study, we conducted a first RNA sequencing run on one replicate of PBS, LPS and poly(I:C) treated mice with the intention to perform quality control analyses. We analyzed the expression levels of cell surface markers CD45, Sca1, CD105 and CD140b used for the prospective isolation of the different non-hematopoietic BM cell types. All analyzed genes showed the expected distribution among cell population, except for CD45 which was elevated in the quintuple-negative cell population suggesting that this population contains to some extent also hematopoietic cells. In addition, we also examined the expression levels of different housekeeping genes among all cell types in all conditions and could see that they are evenly expressed. We then also analyzed the expression of *Kitl* and *Cxcl12* demonstrating that expression of these genes is

largely restricted to ECs and CARs thereby confirmig previously published data [64, 71-73]. Principal component analysis indicated that samples of the same cell types of different conditions clustered tightly with the exception of AECs and SECs. This might be due to a not restrictive enough gating strategy during sorting. Alternatively, Sca-1 – a known Interferon-inducible gene – becomes up-regulated after LPS and poly(I:C) treatment and consequently SECs exhibit higher Sca-1 expression during inflammatory conditions as opposed to steady-state and become less well separable from AECs. Thus, a more restrictive gating will be applied for future experiments.

In summary, we were able to establish a sorting strategy for different non-hematopoietic BM cell populations from different conditions with subsequent RNA extraction and RNA sequencing gave us promising results in quality and quantity controls. With the confidence that our newly established protocol fulfils the expectations of providing enough RNA with a good quality, we are planning to sort AECs, SECs, MSCs, CARs and Unkn from LPS and poly(I:C) treated adult WT mice and from young and aged animals. Subsequently, all samples will be sequenced and the data will be analyzed of differential gene expressions of young, old and inflammation-induced mice. These future experiments hold the promise to be informative on the similarities and distinctions between non-hematopoietic BM stromal cell subpopulations in their hematopoiesis-supporting capacity during ageing and inflammation.

Having established the prospective isolation method for non-hematopoietic BM stromal cell subsets, we chose to analyze gene expression changes of twelve candidate hematopoietic growth factors and inflammatory cytokines with well known functions in steady-state and inflmmation-induced hematopoiesis. Kitl, FLT3L, TPO, GM-CSF and G-CSF are important factors in the regulation of HSCs and HSPCs. The continuous expression of *Kitl* is required for the maintenance of normal hematopoiesis which is achieved by binding to its receptor cKIT which is expressed on all HSPCs [142]. FLT3L is a growth factor for HSPCs and induces HSPC mobilization and is important in dendritic cell (DC) homeostasis [143, 144]. TPO is involved in HSC maintenance during steady-state and supports HSC expansion after transplantation [145, 146]. G-CSF and GM-CSF initiate proliferation and differentiation of HSPCs into mature granulocytes and monocytes. G-CSF is also used in clinics to mobilize HSCs into PB [147] and is highly upregulated during

bacterial infection to induce emergency granulopoiesis [56]. The other analyzed cytokines were known to have a functional role during infection. INFα and INFβ are type I interferons and are essential in the anti-viral response [148]. They are secreted by infected cells and induce an anti-viral state in neighboring cells to limit the spread of the virus. In addition they help to balance the innate immune reaction and activate the adaptive immune system by promoting the development of antigen-specific T and B cells [148]. TNFα is involved in pro-inflammatory responses to systemic inflammation and is one of the cytokines that make up the acute phase reaction [101]. TNFα is not only involved in inflammation but also in processes like cellular communication, cell differentiation and cell death [149]. In addition, also different interleukins were analyzed, i.e. IL-1α, IL-1β, IL-3 and IL-6. IL-1 and IL-6 are like TNFα involved in the acute phase response and are pro-inflammatory signals [101]. IL-6 can also act as anti-inflammatory cytokine depending on the context and has many more described functions like maturation of B cells into antibody-secreting plasma cells [102, 103] and T cell expansion and activation [104-106, 150]. IL-6 was also shown to be important in the immune response against viral or bacterial infections and survial and self-renewal of HSPCs [111, 112]. The two subtypes of IL-1 are distinctly regulated but show similar biological activities like T cell differentiation [151]. IL-3 has similar functions on hematopoiesis like GM-CSF and therefore is acting on progenitor cells to regulate the development into multiple lineages and hence also supports growth and differentiation of T cells as immune response [152, 153]. Although function and expression of these twelve cytokines have been studied extensively, little is known about their primary cellular source during inflammatory conditions. Our RT-qPCR-based expression analysis could confirm some previously published results. For example, *Kitl* was found to be expressed mainly by ECs and CARs in steady-state [73]. Furthermore, *Csf3* was highly upregulated in AECs after LPS treatment [56]. Remarkably, we did not observe relevant upregulation of *Il1β, TNFα* or *Infβ* in any of the non-hematopoietic BM stromal cells after LPS or poly(I:C) treatment although these are all target genes of the NFkB pathway [154, 155]. NFkB can be activated by many different bacteria and viruses and hence regulates the expression of inflammatory cytokines [155, 156]. However, the activation of these target genes of the NFkB pathway has mainly been studied in cells of the immune system but not in non-hematopoietic BM cells possibly explaining this observation.

The most prominent result from our initial gene expression analysis in non-hematopoietic BM stromal cells was the high upregulation of *Il6* specifically in CAR cells after LPS treatment but not poly(I:C) treatment. As IL-6 is a pleiotropic cytokine with many described functions, we focussed our efforts on IL-6 and set out to unravel its regulatory role on the hematopoietic system during gram-negative bacterial infection, mimicked by LPS administration.

To determine whether CAR cells are a relevant source of IL-6 we first examined *Il6* transcription in major solid organs and total bone upon LPS treatment. Surprisingly, we observed that *Il6* was significantly highest up-regulated in total bone as compared to the other organs. We then also analyzed IL-6 protein levels in plasma and BM lysates of reciprocal $Il6^{-/-}$ BM chimeras where *Il6* expression is restricted either to hematopoetic (WT → $Il6^{-/-}$) or non-hematopoietic cells ($Il6^{-/-}$ → WT). We could observe highly elevated IL-6 protein levels in plasma and BM upon LPS treatment only if non-hematopoietic cells were *Il6*-competent indicating that a non-hematopoietic BM cell type is the major source of IL-6. To further investigate whether this cell type might be indeed CAR cells as suggested by our gene expression analysis from prospectively isolalated non-hematpoietic BM stromal cells, we bred *Lepr-cre* mice, which relative specifically target CAR cells [73, 77], with $Il6^{fl/fl}$ mice to generate animals deficient in IL-6 production in CAR cells. The IL-6 protein levels in this *Lepr-Cre;*$Il6^{fl/fl}$ mice were completely abrogated after LPS treatment compared to control mice revealing that CAR cells are indeed the major source of IL-6.

If we followed IL-6 protein levels during chronic-repetitive LPS administration over a period of 3 weeks, we were able to detect increased IL-6 levels, however, to a lower extent than after the first injection. A possible explanation could be a mechanism called endotoxin tolerance (ET), which can occur during chronic bacterial infection. ET is a protective mechanism to prevent excessive inflammation that could lead to a septic shock and has mostly been studied in phagocytic immune cells. Monocytes and macrophages enter an unresponsive state after repeated exposure to endotoxins (e.g. LPS) and hence show an inability to respond to further LPS challenges [140, 141]. Many inflammatory cytokines like TNFα, IL-6, IL-12, IL-1β, CCL3, CCL4 and CXCL10 are downregulated after recurring exposure to LPS [141, 157]. Also other myeloid cells like DCs and

neutrophils have been shown to undergo ET and these cells also downregulate inflammatory cytokines such as TNFα, IL-6 and IL-12. However, whether ET also occurs in CAR cells after repetitive LPS treatment remains to be clarified. Importantly, despite the observed decline in the magnitude of IL-6 protein levels, these remain significantly elevated in LPS-treated mice compared to control animals and thus, will likely be biologically active.

Having confirmed that CAR cells are the major producer of IL-6 after LPS stimulation, we wanted to study the functional relevance of IL-6 during emergency hematopoiesis. Hence, we used a well-established model of emergency granulopoiesis. WT and *Il6* $^{-/-}$ mice were injected twice with LPS in a 48 hour interval and analyzed 24 hours after the last injection. We assessed relative and absolute changes in HSPC and myeloid cell numbers between LPS treated WT and *Il6*$^{-/-}$ mice, but no differences were detected demonstrating that IL-6 is dispensable for emergency granulopoiesis at least during short-term LPS-induced inflammation.

These results prompted us to investigate whether IL-6 would only become relevant for emergency hematopoiesis during chronic-repetitive LPS-induced inflammation. Therefore, we injected WT and *Il6*$^{-/-}$ mice 9 times over a period of 3 weeks and analyzed HSPC and myeloid cell numbers 24 hours after the last injection. We could observe that LPS-treated *Il6*$^{-/-}$ mice had a significantly attenuated increase in their absolute cell numbers of HSCs and HPCs. Moreover, GMPs and more mature myeloid cells even decreased in absolute numbers following chronic-repetitive LPS stimulation. Therefore, mice deficient in IL-6 fail to sustain an adequatetely quantitative emergency hematopoietic response during chronic inflammation-driven hematopiesis. In order to investigate whether the lack of CAR cell-produced IL-6 is responsible for the failure in generating adequate amounts of HSPCs and mature myeloid cells during chronic-repetitive LPS-induced inflammation, we repeated the long-term LPS injections with *Lepr-Cre;Il6*$^{fl/fl}$ mice and their respective controls. We could observe the exact same failure of maintaining a hematopoietic response upon chronic-repetitive LPS stimulation. Importantly, to not only quantitatively assess numbers of immunophenotypically-defined HSPC and myeloid cells populations but also functionally test the role of IL-6 in HSPC biology during chronic inflammation, we performed CFU assays to test HSPC functionaltiy *in vitro*. To this end, we subjected total BM of *Il6*$^{-/-}$ and *Lepr-Cre;Il6*$^{fl/fl}$ mice upon

chronic-repetitve LPS-treatment and their respective controls to fully cytokine-supplemented CFU assays. As expected, we could observe a significant increase in total CFUs in LPS-treated control animals compared to PBS treated mice mainly due to a high increase of CFU-Gs. Not only was there no such increase detectable in LPS-treated *Il6* $^{-/-}$ mice or *Lepr-Cre;Il6*$^{fl/fl}$ mice compared to PBS treated animals but there was even a decrease in CFU activity. Collectively, these data clearly demonstrate that CAR cell-produced IL-6 is required for emergency hematopoiesis and sustained hematopoietic output from HSPCs during chronic-repetitive inflammation.

IL-6 is known to promote hematopoiesis [112] but detailed insights into its mode of action are lacking. Using novel technologies, two recent papers have expanded our knowledge of the role of IL-6 during inflammation-induced emergency hematopoiesis [39, 57]. Zhao et al. investigated the response of single HSPCs toward TLR agonists *in vitro* using a proteomic approach. HSPCs were shown to respond to TLR agonist treatment with the secretion of a variety of inflammatory cytokines. Rigorous functional validation of this interesting finding was hampered by the unavailability of clean experimental methods to specifically and most importantly, exclusively ablate *Il6* from only HSPCs but leaving differentiated myeloid progeny unaffected. The authors therefore had to circumvent this problem by using very specific experimental conditions consisting of transplanting BM from WT or *Il6*$^{-/-}$ donor mice into severely leukopenic hosts devoid of endogenous HSPCs followed by LPS stimulation. Under these experimental conditions, transplanted donor WT HSPCs were more efficient to generate myeloid offspring as compared to HSPCs from *Il6*$^{-/-}$ donor mice. By contrast, our findings unequivocally demonstrated that CAR cells are the major source of IL-6 upon LPS stimulation. These contradictory findings are most likely due to the different experimental approaches used. The findings by Zhao et al. suggest that HSPC-derived IL-6 may play a role in compensating for growth factor deficiencies resulting from a transiently dysfunctional BM microenvironment following recovery from myeloablative hematopoietic stem cell transplantation. Notably, our findings, however, studying naturally-occurring chronic infection / inflammation clearly reveal that IL-6 levels following chronic-repetitive LPS stimulation primarily depend on non-hematopoietic CAR cells.

Moreover, another recently published study described that IFN released from cytotoxic T cells during viral infection acts on BM MSCs leading to secretion of IL-6 which, in turn, promotes monopoiesis. It needs to be noted that MSCs and CAR cells are distinct cell populations and different types of inflammation (viral vs. bacterial) were used in the study by Schuerch et al. and ours. Nevertheless, in order to test whether T cells might be involved in IL-6 release from CAR cells following LPS stimulation we injected WT and Rag1$^{-/-}$ mice, which lack B and T cells due to a defect in immunoglobulin and TCR rearrangement, with LPS and PBS as control and measured systemic and local BM IL-6 levels. We still observed indistiguishable levels of IL-6 in LPS treated Rag1$^{-/-}$ mice compared to LPS treated WT animals clearly demonstrating that T cells are dispensable for IL-6 secretion from CAR cells upon LPS-induced inflammation.

Having shown that IL-6 plays a critical role in sustaining hematopoiesis following chronic-repetitive LPS-induced inflammation, we set out to determine whether IL-6 acts directly or indirectly onto HSPCs to stimulate enhanced proliferation and differentiation. Although we cannot definitively prove direct IL-6 action on HSPCs, two lines of evidence suggest that this may indeed be the case. First, we found detectable expression of both CD126 (i.e. IL-6Rα) representing the ligand-binding subunit of the IL-6R as well as CD130 (i.e. gp130) representing the signal-transducing subunit on HSPCs by FACS. Second, c-Kit-enriched BM cells from WT mice subjected to CFU assays and grown in presence of IL-6 generated more myeloid colonies than control cells cultured in the absence of IL-6.

Finally, we investigated whether LPS binds directly to CAR cells to activate IL-6 production and secretion or whether LPS sensing occurs in a different cell population which, in turn, stimulates IL-6 release from CAR cells indirectly. Therefore, we assessed Toll-like receptor expression in CAR cells by RT-qPCR and found *Tlr4*, the cognate receptor for LPS, to be highly expressed on CAR cells demonstrating that CAR cells are in principle able to directly sense LPS and respond with an increased IL-6 secretion. In addition, we also measured IL-6 protein levels after LPS treatment in reciprocal *Tlr4*$^{-/-}$ BM chimeras in which *Tlr4* expression is restricted to either hematopoetic (WT → *Tlr4*$^{-/-}$) or non-hematopoietic(*Tlr4*$^{-/-}$ → WT) cell types. After a single LPS injection, whereas local BM IL-6 levels depended on both TLR4 on hematopoietic as well as non-hematopoietic cell types,

systemic IL-6 levels fully depended on TLR4 on non-hematopoietic cells. By contrast, following chronic-repetitive LPS stimulation over 3 weeks systemic and local BM IL-6 levels were completely depended on TLR4-expressing non-hematopoietic cells. Although multiple non-hematopoietic cell types express TLRs [55] and no currently available experimental model system allows specific deletion of *Tlr4* in CAR cells our above-described data suggest that CAR cells indeed integrate LPS sensing and IL-6 production within one cell type.

In summary, we could demonstrate using different mouse models that CAR cells are the major producer of IL-6 during LPS stimulation and that IL-6 is necessary to sustain emergency hematopoiesis during chronic repetitive LPS stimulaiton. Importantly, our findings further emphasize that the BM microenvironment not only plays a critical regulatory role in steady-state but also during infection- and inflammation-driven hematopoiesis. A better understanding of the mechanisms operating during the hematopoietic system's adaptation to infection and inflammation may hold the promise to devise new therapeutic interventions to treat infectious and inflammatory diseases.

Conclusions

1. BM CAR cells are the primary source of systemic and local BM IL-6 levels after LPS stimulation.
2. IL-6 is dispensable for emergency hematopoiesis during short-term LPS administration.
3. IL-6 is required for an adequately quantitative increase in numbers of immunophenotypically-defined HSPCs and differentiated myeloid cells during chronic-repetitive LPS-induced inflammation.
4. IL-6 is essential to increase functional CFU activity during chronic-repetitive LPS-induced inflammation
5. HSPCs express the IL-6 receptor α and β subunits and have therefore the ability to respond to IL-6 via *cis*- or *trans*-signaling.
6. BM CAR cells express multiple Toll-like receptor genes including *Tlr4,* which renders them capable of direct LPS sensing.
7. Systemic plasma and local IL-6 levels depend on Tlr4-expressing, non-hematopoietic cells.

Material and Methods

Mice

In this study C57BL/6J (Janvier) mice were used. All animals were maintained at the University Hospital Zurich animal facility and treated in accordance with guidelines of the Swiss Federal Veterinary Office. Experiments and procedures were approved by the Veterinäramt des Kantons Zurich, Switzerland.

LPS and pIC injections

Mice received 2 intraperitoneal injections of 35 µg ultrapure LPS from E. coli 0111: B4 (InvivoGen) or 100 ug poly (I:C) (InvivoGen) 48 hours apart and non-hematopoietic BM cell populations were prospectively isolated 4 hours after the last injection.

Processing of bones

Femurs, tibias and hip bones were removed, BM was flushed and cells were resuspended with digestion medium (DMEM/10% FCS/10mM HEPES/0.4% collagenase II (Worthington)/0.02% DNase I (Worthington)). Bones were cut into small pieces and combined with flushed BM in a total of 5 ml digestion medium. Bones and BM were incubated for 45 min at 37°C on a shaker and afterwards the cell suspension was filtered through a 70um cell strainer. LS MACS columns (Miltenyi Biotec) were then used to enrich the cell suspension for $CD45^{-}Ter119^{-}$ cells following the supplier's protocols.

Flow cytometry

The following antibodies (all from eBiosciences, unless otherwice stated) were used to assess and sort different non-hematopoietic BM stromal cell types: anti-Sca1 (D7, Biolegend), anti-Ter119 (TER-119, Biolegend), anti-CD45 (30-F11, Biolegend), anti-CD31 (390), anti-CD105 (MJ7/18), and anti-CD140b (APB5).

RNA extraction

AEC, SEC, MSC, CAR and Unkn cell populations were sorted directly into RLT Lysis Buffer provided by Qiagen (RNeasy Micro Plus Kit). RNA was extracted following the supplier's protocols. RNA quantities and qualities were measured using the 2200 TapeStation (Agilent Technologies).

Sample preparation and Microarray analysis

The WT-Ovation One-Direct System (Nugen) and Encore Biotin Module (Nugen) were used for the synthesis of cDNA and its fragmentation following the supplier's protocol. The microarray analysis was performed on a Affymetrix Mouse Gene 2.0 ST Array chip containing 35'240 gene probes.

RNA Seq library preparation and Sequencing

600 pg of input total RNA were used for cDNA synthesis and the library preparation with the Clonetech SMRTer Stranded total RNA-Seq Kit for Pico Input Mammalian Total RNA. The library was sequenced using the Illumina HiSeq4000 instrument.

Additional Material and Methods used for the Research article are listed in the section "Research article".

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